

# ANNUAL REVIEW OF BIOCHEMISTRY

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## PREFACE

In the introductions to the three preceding volumes we have described the circumstances leading to the inception of the *Review* and the considerations which seemed of major importance in the development of editorial policy. Most noteworthy are the unabated growth in the field as a whole, the ever-rising tide of published work urgently in need of integration and synthesis, the dramatic suddenness with which new discoveries illuminate old and vexing problems, and the tendency of other once-active phases to become quiescent.

The experience of the past three years convinces us of the futility of attempting to encompass the whole of this vast literature within a single volume and of adhering rigidly, year by year, to a set list of topics. Though at the expense of many omissions we trust that readers will regard with favor the present policy of encouraging, on the part of reviewers, analysis of selected papers, constituting only half or less of those published, but chosen on the basis of their intrinsic importance or of their significance in relation to problems under discussion. Consequently, it is evident that many valuable contributions must be placed aside, perhaps only temporarily, because they are too isolated in nature to be woven into a coherent chapter.

In so fluid a subject as biochemistry, the dangers of stressing certain phases unduly, and of failing to take cognizance of other fields of importance are ever imminent. To lessen such dangers we feel obliged to depend more and more upon the judgment and experience of those who use the *Review*, hoping that we may frequently receive their critical comments and suggestions in respect to subject matter as well as to other questions pertinent to the usefulness and availability of the *Review*.

In accordance with the policy of giving due recognition to fields of immediate interest we are glad to include within the present volume reviews on choline and allied compounds by Professor Gaddum, on the biochemistry of malignant disease by Mrs. Holmes, and on plant hormones by Professor Thimann. Reviews on soil bacteriology, biochemistry in relation to selected problems in medicine, and on the application of X-ray methods to the elucidation of the structure of compounds of biochemical interest will appear in Volume V (1936).

We cannot cease to acknowledge the debt of gratitude, expressed by many readers of the *Review*, to those who have given of their time

and patience in the writing of these reviews. The judicious selection of papers appropriate for survey, and their critical analysis and integration into compact reviews, rigorously limited in length, are tasks of unusual difficulty. The cordial reception which the *Review* has enjoyed and its widening distribution from year to year are due entirely to the generous co-operation of its contributors.

With the present volume of the *Review* the introductory three-year period comes to an end and our duties as editors become expanded into those of publisher as well. The enterprise is now established as a non-profit corporation and shall be administered with the object in mind of adding to the quality, usefulness, and availability of the *Review*. In undertaking these added responsibilities we shall endeavor to be fully mindful of the interests of those who use the *Review* and responsive to their criticism and advice.

It is again a pleasure to express our appreciation of the painstaking care of Stanford University Press in printing the volume and of its cordial assistance throughout.

C. L. A.  
D. R. H.  
J. M. L.  
C. L. A. S.

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## PERMEABILITY\*

By M. H. JACOBS

*University of Pennsylvania, Philadelphia*

In order that this review of the published work on permeability for the greater part of the years 1933 and 1934 may be kept within the allotted limits, it will be necessary to confine it to the more general aspects of the subject, omitting for the most part the relatively complicated activities of the organs of the higher animals such as the kidney, intestine, etc., as well as the intake of materials by the higher green plants, which is dealt with separately in a later section. The special subjects chosen for discussion are those which have been most prominent in the literature of the past two years; others of perhaps equal interest but represented by less published work have, with reluctance, been excluded for lack of space.

### FACTORS CONCERNED IN THE ENTRANCE OF MOLECULES INTO CELLS

Most of the work in this field has had to do with the relative importance of two factors: lipid solubility and molecular volume. A very extensive and important contribution to the subject is that of Collander & Bärlund (1) on the permeability of the large multi-nucleate cells of *Chara ceratophylla*. This work is noteworthy not merely because of the large number of substances, forty-five in all, whose behavior was studied, but because the measurements of cell permeability were made by the direct method of chemical analysis of the cell sap, which involves a minimum of theoretical assumptions, and also because the data are presented in a form that permits accurate quantitative comparisons with similar data for other types of cells.

The general results obtained confirm the earlier conclusions of Collander & Bärlund and other workers, reached by different methods with different types of material, that both lipid solubility and molecular size are factors of importance in cell permeability. Thus, the highly lipid-soluble triethylcitrate penetrates with great rapidity in spite of its large molecular volume, while methyl alcohol, whose molecule is the smallest of all those studied, penetrates even more rapidly in spite of a relatively low lipid solubility. In a series of closely related compounds such as urea and its methyl-, ethyl-,

\* Received January 29, 1935.

dimethyl-, and diethyl-derivatives, or trimethyl- and triethyl-citrates, or glycerol, monacetin, and diacetin, the factor of lipid solubility so dominates the situation that the rate of penetration in each case increases rather than decreases with increasing size of the molecule. In the series: ethylene-glycol, glycerol, erythritol, mannitol, on the other hand, the relatively enormous observed differences in permeability are probably attributable to the sizes of the molecules, the lipid solubility of all being very low. A rather characteristic situation is presented by the series of amides, in which the rate of penetration from acetamide to *dl*-valeramide, inclusive, varies directly with the lipid solubility and inversely with the molecular volume, but in which the lowest member, formamide, has a sufficiently small volume so that its penetration occurs more readily than that of several of its more lipid-soluble relatives.

Similar studies have been made by Bonte (2) on cells representing the algae (*Hydrodictyon*), the fungi (*Basidiobolus*), and the mosses (*Hookeria*). Direct chemical analyses of the cell sap being impracticable in these cases, indirect osmotic methods were employed, and Lepeschkin's constant,  $\mu$ , was used as a measure of permeability. This constant is defined by the relation  $\mu = 1 - K/K'$ , where  $K'$  represents the critical plasmolytic concentration for a given penetrating substance and  $K$  the similar concentration for a non-penetrating substance. The permeability values obtained in this way, though less satisfactory than those of Collander & Bärlund, illustrate the same principles, the most lipid-soluble substances being found to penetrate all three cells with ease, and those showing little lipid solubility penetrating for the most part according to their molecular volumes.

Another study of the same sort was made by von Hofe (3) on cells from the stalk of the common mushroom, *Psalliota campestris*, Lepeschkin's constant,  $\mu$ , also being used as a measure of permeability. Again, evidences of the importance of both lipid solubility and of molecular volume were obtained, but the cells of *Psalliota* proved to have an unusually low permeability to substances of the relatively lipid-insoluble type. An interesting example of the importance of molecular volume as a factor in the permeability of cells of the higher plants was found by Höfler (4) in the case of epidermal cells from *Gentiana Sturmiiana*, the behavior of these cells in many respects suggesting that of *Beggiatoa*, the classical example of a cell to which a "molecular sieve" theory of cell permeability seems to be applicable.

In the case of animal cells, the importance of molecular volume also appears. For example, Schmengler & Höber (5) compared the rate of escape of various substances, nearly all of low lipid solubility, from the kidney of the frog, when the latter was perfused from the renal portal vein. Special experiments showed that the behavior of these substances, unlike that of phenol red, was little influenced by narcotics, and it was therefore concluded that processes of secretion or reabsorption were not involved. The following substances (each accompanied by its relative, calculated, molecular volume) were studied: ethylene glycol (65.5), acetamide (68.7), thiourea (69.6), methyl urea (81.2), glycerol (87.8), dihydroxyacetone (93.8), lactamide (98.5), malonamide (104.4), butyramide (112.7), creatinine (115.7), asparagine (134.2), glucose (183.2), and mannitol (189.2). In this series the effect of molecular volume was very clearly shown. Thus, asparagine, glucose, and mannitol, as far as could be determined, did not penetrate the wall of the tubule at all, creatinine and malonamide did so with difficulty, and the substances with smaller molecular volumes appeared in the urine in quantities that in general increased with decreasing size of the molecule. As would be expected, butyramide, which is somewhat lipid soluble, showed a higher rate of penetration than that predictable from its molecular volume alone.

An exception to the relation between molecular volume and permeability is furnished by the behavior of glycocoll and other amino acids, studied by essentially the same method by Robbins & Wilhelm (6) and further discussed by Schmengler & Höber (5). Though glycocoll has a calculated molecular volume of 76.5, which is less than those of methyl urea (81.2), glycerol (87.8), lactamide (98.5), and creatinine (115.7), it fails to pass from the blood across the tubule cells in the same manner as the other substances. While amino acids, like sodium chloride and glucose, are strongly reabsorbed from the lumen of the tubule and simple physical diffusion in the opposite direction may for this reason be difficult, a further possibility is suggested by the observations of Schmengler (7) on the permeability of dried collodion membranes, which showed that in its rate of penetration, glycocoll (76.5) lies between glycerol (87.8) and erythritol (130.2). Similarly, alanine (98.5) shows approximately the same rate as arabinose (153.4), while leucine (164.5) penetrates somewhat more slowly than mannitol (189.2). These and other similar facts are explained by Schmengler as being due to the highly polar properties of the amino acids and the resulting orientation of the

surrounding water dipoles. In the case of the salivary gland of the cat, also recently studied by Schmengler (8), the behavior of glycocoll is even more aberrant.

Another case in which molecular size has been related to permeability is that of the passage of proteins across the glomerular membrane in the mammalian kidney [Bayliss, Kerridge & Russell (9)]. According to this investigation, gelatin, Bence-Jones protein, and egg albumin are able to pass the membrane in question, while serum albumin and globulin, edestin, casein, and hemocyanin are retained; hemoglobin falls about on the dividing line, escaping into the urine when its plasma concentration exceeds a certain level. The proteins which are readily excreted all have molecular weights below 70,000, while those which are retained have higher values; hemoglobin, with a molecular weight of 68,000, is intermediate. The behavior of hemoglobin is also illustrated by the work of Amberson and his associates (10) who found that one of the physiological defects of a hemoglobin-Ringer solution as a substitute for blood is the gradual loss from it of hemoglobin through the kidneys. The rate of this loss in the frog has been shown (11) to depend to a very considerable extent on the pH of the perfusion medium, decreasing greatly as the medium becomes more alkaline. By immunological methods, Ratner & Gruehl (12) have obtained evidence of the passage of unsplit proteins across the intestinal wall of mammals, but little is known as to the exact mechanism of this passage.

#### SPECIFIC DIFFERENCES IN PERMEABILITY

Several examples of specific differences in the permeability of plant cells are given by Höfler (4). Though the behavior of the substances studied in general follows the principles discussed in the preceding section, the exact order of penetration varies considerably from cell to cell. Very striking specific differences are also shown by the unpublished work of Hofmeister (quoted by Höfler in the same paper) in which the behavior of urea and of glycerol is compared in the case of seventeen species and that of urea and of eight other substances in the case of five species of plants.

Similar conditions have been known for some years in the case of the erythrocytes of the vertebrates. A recent study of importance in this field is the systematic comparison by Höber & Ørskov (13) of the rates of penetration of fifteen substances into the erythrocytes of nine species of vertebrates. Though the data obtained are too exten-



sive to summarize here, they leave no doubt as to the highly specific nature of permeability in this particular type of cell.

An extension of these studies has recently been made by Ulrich (14) [see also Höber & Ulrich (15)] in the case of substances of very low lipoid solubility and relatively high molecular volume, which enter most cells only with the greatest difficulty, but which show a highly specific ability to penetrate some kinds of erythrocytes. Two striking cases of this sort were previously known: the permeability of the erythrocytes of man and the apes to glucose; and that of the mouse, and to a much less extent that of the rat, to erythritol. Ulrich & Höber have shown that these peculiarities are associated not merely with the individual substances mentioned but with groups of chemically related substances. Thus, the erythrocytes of man, which are permeable to glucose, are even more permeable to arabinose and xylose, to which the erythrocytes of most species are practically impermeable. The erythrocytes of the mouse, on the other hand, are not only very freely permeable to erythritol but they show a highly specific permeability to the much larger molecules of the related compounds, mannitol and iso-dulcitol. In other words, the erythrocytes of man seem to be more or less specifically permeable to certain sugars, and those of the mouse to certain polyhydric alcohols. Furthermore, the erythrocytes of the guinea pig are shown to possess a permeability to amino acids which is unique among the species studied. It is interesting to note that of the seven species of mammals investigated three should show highly characteristic departures from the usual principles of cell permeability. Results such as these suggest that the "lock-and-key" mechanism frequently postulated for enzyme action may conceivably play a part in cell permeability as well. An additional fact, possibly related to those just discussed, but the full significance of which is not yet clear, is the observation of Jacobs & Corson (16) that minute traces of copper have a specific and reversible effect in reducing the unusually great permeability of the erythrocytes of man and the rat to glycerol to a level comparable with that found normally in other species.

#### PERMEABILITY TO ELECTROLYTES<sup>1</sup>

The ease with which certain weak electrolytes enter living cells is illustrated by the work of Beck (17) who has compared the effec-

<sup>1</sup> Cf. also this volume, pp. 436, 527. (EDITOR.)

tiveness of carbon dioxide and ammonia, on the one hand, and of hydrochloric acid and sodium hydroxide on the other, when added to sea water, in producing certain characteristic changes in the oxidation-reduction potentials in starfish eggs. Blinks (18) has also demonstrated the ability of the small amounts of free ammonia present in solutions of ammonium salts to enter *Halicystis* cells, thereby producing an increased alkalinity of the cell sap, and, under appropriate conditions, a reversal of the potential difference across the protoplasm. Wehrli-Hegner & Wyss (19), using closed portions of veins of the frog into which antimony electrodes were introduced, have shown that changes in internal pH are produced far more readily by carbon dioxide than by the stronger acids, hydrochloric, sulphuric, and phosphoric. Of particular physiological interest is the fact that an increased internal acidity could be demonstrated when the external solution was pure bicarbonate. A special quantitative study of the rate of diffusion of carbon dioxide in animal tissues has been made by Wright (20). Another study of relatively weak electrolytes is that of Bodansky (21) on the hemolytic powers of fatty acids. Though the rate of hemolysis doubtless depends to some extent on factors other than permeability, the results obtained are in good agreement with those of other workers on the penetrating powers of similar homologous series of organic compounds.

Of particular interest is the recent work on the permeability of cells to strong electrolytes. An important series of studies in this field is that of Osterhout (22) and his associates on the intake of  $K^+$  and other cations by the large multinucleate cells of *Valonia*. In confirmation of their earlier but less extensive work, Jacques & Osterhout (23) have found that an increase of the pH of the surrounding sea water increases the rate of entrance of  $K^+$  and vice versa. They picture the mechanism of penetration as involving potassium hydroxide, either as such, or after combination with some constituent of the cell surface, which they believe to be impermeable or nearly so to ions, to form a soluble compound. According to this theory, the tendency of  $K^+$  to enter the cell depends not simply on its own external concentration but rather on the external product,  $[K^+][OH^-]$ . Since its tendency to escape from the cell depends on a similar internal product, an equal distribution is not to be expected when a difference in reaction exists between the cell sap and the surrounding medium, which is the usual condition in living cells. As further evidence in support of this view Jacques & Osterhout have shown that when the product  $[K^+][OH^-]$

becomes greater in the interior of the cell than externally, either through an increased internal alkalinity or a decreased external potassium concentration or alkalinity,  $K^+$  may leave the cell even while  $Na^+$  continues to enter.

Though the same results might be obtained by an exchange of  $H^+$  for  $K^+$  ions through a membrane permeable to cations, Jacques & Osterhout consider this explanation to be unlikely because of the numerical results obtained by substituting experimentally determined data for two external pH values in the following expressions (in which the subscripts  $o$  and  $i$  indicate outside and inside, respectively):

$$[K^+_o] [OH^-_o] - [K^+_i] [OH^-_i] \quad (a)$$

$$[K^+_o] [H^+_i] - [K^+_i] [H^+_o] \quad (b)$$

Of these,  $a$  is considered to be a measure of the rate of accumulation of  $K^+$  if KOH enters the cell and  $b$  if  $K^+$  is exchanged for  $H^+$ . For an actual case of this sort in which the initial values both inside and outside of  $[K^+]$ ,  $[OH^-]$ , and  $[H^+]$  were known, the theoretical ratio of the calculated rate at pH 8.8 to that at pH 8.2 proved to be 5.53 by mechanism  $a$  and 1.09 by mechanism  $b$ . Since the observed average ratio for a number of experiments was 3.5, this result is considered to support the solubility theory, particularly since the calculated value 5.53 would be somewhat reduced by making allowance for changes due to photosynthesis. Further arguments against the ionic exchange theory are given elsewhere by Osterhout, but limitations of space prevent their discussion here.

Though it seems plausible to suppose that one or the other of the two mechanisms just mentioned may in some way be involved in the intake of potassium by *Valonia*, certain difficulties should not be overlooked. The first is the fact that in the absence of photosynthesis the potassium intake ceases, even though the relation of the two products,  $[K^+] [OH^-]$ , remains favorable. Several possible explanations have been offered to account for this fact, but whether they will prove to be adequate can be determined only by further work. A second difficulty is that in *Valonia*, potassium is stored chiefly as potassium chloride rather than as potassium hydroxide or as a salt of an organic acid produced by metabolic processes. Evidently, therefore, chloride must enter the cell with approximately the same ease as potassium. Since the direct intake of ions in considerable quantities is ruled out by his general theory of permeability, Osterhout

supposes that by the penetration of potassium hydroxide there are first formed potassium salts of carbonic or other organic acids, such as are, in fact, known to appear in the sap of many plants (24). Later, it is supposed, by the entrance of undissociated hydrochloric acid, the salts first formed are converted into chlorides, with the escape of the free acid. While this hypothesis carries to its logical conclusion the conception of a cell surface practically impermeable to ions, it is not free from difficulties when an attempt is made to picture the exact details of the mechanism involved.

The entrance of potassium into several types of animal cells has also been studied by other workers. It was originally shown by Loeb & Cattell that the heart beat of the developing *Fundulus* embryo is stopped by placing the egg in a solution of a potassium salt; this stoppage is reversible, and the escape of potassium from the poisoned heart is favored by acidity of the external solution. Bridges & Sumwalt (25) have recently studied the opposite process, and have found that within certain limits poisoning is made less rapid by increasing the acidity of the external solution. The simplest interpretation of these results is that the movement of potassium in either direction is favored by a high value of  $[K^+]$   $[OH^-]$  on the side from which diffusion is proceeding and by a low value on the opposite side. Bridges & Sumwalt do not attempt to decide whether the mechanism involved is a passage of potassium hydroxide as postulated by Osterhout for plant cells or an exchange of potassium for hydrogen ions. In favor of the latter view, however, is the fact, also originally observed by Loeb & Cattell, that the escape of potassium is favored not only by acids but by other electrolytes as well.

The behavior of potassium in muscle has received particularly thorough treatment. It has long been known that it is normally present in muscle cells at a far higher concentration than in the blood and lymph, and various lines of evidence indicate that it exists there chiefly in an uncombined ionic form. It has also been known that changes in its external concentration bring about prompt changes in its internal concentration, though always with the preservation of large concentration gradients. Fenn and his co-workers (26, 27, 28, 29) have shown that, as in the case of *Valonia*, the equilibrium between the external and internal potassium is greatly influenced by pH changes. Thus, in one experiment it was found that at pH 6.3 the external equilibrium concentration was 70 mg. per cent while at pH 7.7 it was only 10 mg. per cent. Over a certain range, indeed, the external

$[K^+][OH^-]$  product at equilibrium remains fairly constant. An effect of pH changes within the cell can also be demonstrated in some cases. Thus, the addition of ammonium chloride to the external solution tends to produce an internal alkalinity accompanied by an escape of potassium. The addition of carbon dioxide to a muscle surrounded by Ringer's solution causes a greater increase in external than in internal acidity and likewise favors an escape of potassium. Netter (30) has made independent studies of the same sort and reports similar results. For example, monoiodoacetic acid, which has an alkalinizing effect on muscle, causes an escape of potassium.

The relation between internal reaction and internal potassium concentration is, however, by no means simple. Thus, as far as Fenn could determine by an application of the Henderson-Hasselbach equation, the pH of the muscle may change in either direction without any change in the internal potassium concentration and, though the entrance of potassium sometimes produces an increased internal alkalinity, as would be expected if it were exchanged for hydrogen ions or if it entered as potassium hydroxide, this increase is not at all constant. Any simple equilibrium that might otherwise be reached is doubtless complicated by the fact that the entrance of potassium into the cell seems to be associated with an increased oxygen consumption, a breakdown of phosphocreatine, and, in higher concentrations, with a production of lactic acid (29).

Fenn and his associates have not attempted to decide between the theory that potassium penetrates as potassium hydroxide and that it is exchanged for  $H^+$ , though inclining towards the latter explanation. The ionic exchange theory is, however, definitely accepted by Netter (30), who, by a somewhat indirect method of calculation, arrives at the conclusion that the ratio of  $[H^+]_{(inside)}$  to  $[H^+]_{(outside)}$  is not so different from that of  $[K^+]_{(inside)}$  to  $[K^+]_{(outside)}$  as has been generally supposed. While his conclusion that a pH value of 6.2 exists within the normal muscle cell is difficult to reconcile with the results of other workers, he very rightly emphasizes the fact that pH values based on analyses of the muscle as a whole may fail to give a correct idea of the values existing in the regions of most importance for an ionic exchange. Netter has also studied the distribution of the ammonium ion and finds that, like  $K^+$ , it is capable, though perhaps in an indirect manner, of entering the muscle cell against a concentration gradient.

No review of the literature on cell permeability to ions would be

complete without reference to the ingenious model experiments of Osterhout and his associates (24, 31, 32, 33, 34, 35). Unfortunately, space does not permit a description of this extensive work here, but its omission is rendered less serious by the fact that Osterhout (22) has recently prepared an excellent general summary of most of it, in its relation to the behavior of *Valonia*, etc. An important paper dealing with ionic exchanges and equilibria in the alimentary tract of the vertebrates and with model experiments which provide plausible explanations of certain interesting physiological phenomena has been published by Teorell (36), but it is likewise too long to be adequately summarized here. A recent paper by Norberg (37) also deals with a similar subject. Another case of ionic equilibrium having physiological applications is that involved in the ultrafiltration of blood plasma, studied by Ingraham, Lombard & Visscher (38).

#### THE PERMEABILITY OF THE ERYTHROCYTE

In addition to the studies mentioned elsewhere in this review in which erythrocytes were used merely as suitable material for the investigation of general problems of cell permeability, a considerable number of papers is concerned with the specific properties of the cell itself. Andreen-Svedberg (39) has reviewed the literature and presented original data on the permeability of the erythrocytes of different species, but particularly those of man, to glucose. She answers in the negative the much disputed question as to whether glucose distributes itself between the plasma and the erythrocytes in the same proportions as water. Fleischmann & Kaunitz (40) have also studied the permeability of the human erythrocyte to glucose. They confirm the findings of previous workers that whereas chemical methods indicate a rapid absorption of this substance, the volume changes observed by the hematocrit method do not. Instead of interpreting this discrepancy as being due to adsorption of glucose at the surface of the cell, they believe it to result from a loss of water from the cells during their packing in the hematocrit tube. By a different method of volume measurement they have obtained results in good agreement with those found by chemical methods.

Several recent papers deal with the permeability of the erythrocyte to ions. Maizels (41) by chemical methods has studied the rate of uptake of forty different anions, the standard of comparison in each case being  $\text{Cl}^-$ , which was allowed to penetrate simultaneously. Certain general conclusions are drawn from his experiments, such as,

that the ions  $\text{SCN}^-$ ,  $\text{I}^-$ ,  $\text{NO}_3^-$ ,  $\text{Cl}^-$ ,  $\text{SO}_4^{=}$ , and  $\text{PO}_4^{=}$  penetrate in the order of the lyotropic series, that the presence of polar groups in an organic molecule tends to reduce the rate of penetration of its ions, and that factors such as the pH of the solution and the strength and the lipid solubility of the acids from which the ions are derived are of importance. From the nature of the method used and the general complexity of the situation, however, the actual figures obtained can scarcely be considered to have more than a semi-quantitative significance.

The same author (42) has compared the pH changes in suspensions of erythrocytes in solutions of non-electrolytes, of electrolytes and in mixtures of the two. In general, in non-electrolyte solutions, the medium not only gains  $\text{Cl}^-$  but becomes more acid at the same time; electrolytes in the external solution tend to prevent this effect. These results, as well as those of Jacobs & Parpart (43) and of Jacobs, Parpart & Corson (44), who showed that certain types of osmotic hemolysis are opposed by the absence of electrolytes, are explicable on the hypothesis that in a non-electrolyte solution an exchange of internal chloride ions for external hydroxyl ions occurs, which is decreased by the presence of electrolytes, externally. Such an exchange ought theoretically to decrease the internal osmotic pressure of the cell by a shift of base from combination with other acids to hemoglobin.

Ørskov (45), by the use of chlorine, bromine, and iodine electrodes, has studied the permeability of the erythrocyte to the ions of these three halogens. He reports that the exchange of  $\text{I}^-$  for  $\text{NO}_3^-$  from erythrocytes previously brought into equilibrium with a solution of sodium iodide is practically complete in two minutes, and that the similar exchange of  $\text{I}^-$  for  $\text{ClO}_4^-$  requires six or seven minutes, while that of  $\text{Cl}^-$  for  $\text{HCO}_3^-$  requires only one-quarter minute. Devadatta (46) has studied the distribution of lactates between erythrocytes and the surrounding plasma under different conditions, and has found that as in the case of  $\text{Cl}^-$  a lactate shift into the cells is caused by an increased partial pressure of carbon dioxide, a decreased partial pressure of oxygen, or by an increase of acidity.

The much disputed question of the possible permeability of the erythrocyte to cations arises in several recent papers. Ørskov (45, 47) has found that the rate of entrance of ammonium salts into erythrocytes is enormously increased by the presence of carbon dioxide and of bicarbonates. He gives evidence that the rate of exchange of anions under these conditions is little affected and concludes that an



altered permeability of the cell to ammonium ions, as such, is involved. Though he has not overlooked the possible rôle of undissociated molecules of carbon dioxide and ammonia, this possibility has perhaps been dismissed with a less detailed consideration than it deserves.

Another question having to do with the permeability of the erythrocyte to cations arises in connection with the work of Ponder (48) and his collaborators who have explained many of the apparent anomalies in the osmotic behavior of this cell by a relatively rapid escape of salts from its interior. That this theory cannot account for the decreased swelling of erythrocytes and the decreased tendency to osmotic hemolysis at high temperatures is shown by the fact that cells first exposed to high temperatures behave normally when the temperature is subsequently lowered (49). That it probably cannot account for the greater part of the similar effect observed in non-electrolyte solutions is indicated by the ready reversal of the effect (43).

There is still left, however, a considerable amount of evidence that leakage in hypotonic solutions is a factor to be reckoned with. By the hemolysis method indirect evidences of its occurrence over times varying from minutes, for some species, to hours for others are easy to obtain (43), while direct chemical evidence of the escape of potassium from certain erythrocytes during periods of a few minutes has recently been presented by Davson (50) and by Ponder & Robinson (51). It is inferred by the latter authors that since the final volumes of cells in hypotonic solutions are attained within fifteen seconds the escape of salts must also have been completed within that time. If this is true, it becomes necessary to account for the remarkable fact that a cell placed in a hypotonic solution almost instantly loses a definite proportion of its salts, accurately related to the concentration of the external medium, but not determined by a simple diffusion equilibrium, since, as has been shown by previous workers by chemical, electrical, and osmotic methods, further losses continue over long periods. Whatever the final interpretation of these results may be, it is fairly certain that they do not indicate a permeability of the erythrocyte to cations under normal body conditions. The fact that Davson under the conditions of his experiments found almost the same rapid rate of escape of potassium from ox erythrocytes into ox serum as into salt solutions, whereas in the circulating blood this escape obviously does not occur, is an indication of the readiness with which the properties of the erythrocyte may be changed by experimental manipulation.



## THE KINETICS OF PENETRATION

During the past two years a considerable number of cases of cell permeability have received mathematical treatment, the primary object of which has been to provide methods for the quantitative measurement of the rate of cell penetration by water, by solutes, or by both. As regards the uncomplicated penetration of a cell of constant volume by a solute alone, the work of Collander & Bärklund (1) leaves little to be desired. Assuming that Fick's law holds for the passage of substances across the thin protoplasmic portion of the cell and that because of convection currents and more rapid diffusion elsewhere the only significant concentration gradient is that across the protoplasm itself, they derive the following relation:

$$k = \frac{v}{qt} \ln \frac{C}{C - c}$$

where  $v$  is the volume and  $q$  the surface of the cell,  $c$  its internal concentration at the time  $t$ ,  $C$  the equilibrium internal concentration, and  $k$  a constant which measures permeability. That this law is actually followed in the penetration of the cells of *Chara* by many substances is shown by extensive experimental data. It is also shown that the constant which measures the rate of escape of a substance previously brought by inward diffusion to a given concentration in the cell sap is almost the same as that for its rate of entrance. Further points of interest are brought out in the following table in which are compared: (a) the times required for half-saturation of living cells; (b) the corresponding times for dead cells; (c) those calculated from the laws of diffusion for cylinders of water of the same dimensions as the cells:

Substance	Time in Minutes Required for Half-Saturation		
	(a) Living Cell	(b) Dead Cell	(c) Water Cylinder
Methyl alcohol .....	1.3	0.8	0.27
Urea .....	320.0	0.9	0.34
Acetamide .....	24.0	1.2	0.38
Glycerol .....	1,700.0	1.9	0.49
Trimethylcitrate .....	5.5	2.2	0.67
Saccharose .....	50,000.0	4.1	0.92

In the case of the penetration of water alone, a recent contribution of importance is the mathematical paper of Manegold & Stüber

(52), who have derived equations for the osmotic volume changes of spherical and cylindrical cells under a variety of hypothetical conditions. Experimental studies in which the rate of penetration of water alone has been expressed by a definite permeability constant have been made by Kekwick & Harvey (53), who have shown that the permeability of *Arbacia* eggs is decreased in the absence of oxygen, and by Fowler (54) who has studied the effect of the electric current on the permeability of the same eggs. Huber (55) and Huber & Schmidt (56) have also made quantitative studies of the permeability of plant cells to water, but the nature of their constant is not such as to permit accurate comparisons between cells of different sizes.

The more complex case of the simultaneous penetration of a cell by a solute and by water is somewhat simplified when the entrance of the solute is slow in comparison with that of the solvent, and a condition of osmotic balance between the cell and its surroundings may at all times be assumed to exist. Schiødt (57) has studied a case of this sort, namely, the rate of swelling of erythrocytes in solutions of ammonium salts, and has found a good agreement between experiment and theory. Hurch (58), by less accurate methods, has also obtained information as to the relative permeability of different plant cells to solutes, from observations of their volume changes in solutions of slowly penetrating substances.

The most general case, in which the permeability of the cell to water and to a simultaneously penetrating solute may have any relation whatever to each other, has been discussed in several papers by Jacobs (59, 60, 61, 62). For the erythrocyte, tables have been prepared which permit the simultaneous determination of permeability constants for water and for a penetrating solute from observed times of hemolysis. A method is also described for the determination of both kinds of permeability constants from measurements of the minimum volume reached, and the time of its attainment, when a cell is exposed to its natural medium, rendered temporarily hypertonic by the addition of a penetrating solute.

The volume changes of Osterhout's cell model, involving the penetration of both water and a solute, have also been treated theoretically by Osterhout (31) and by Longworth (63). Though both methods of treatment permit calculations to be made which are in good agreement with the observed results, the former leads to the erroneous conclusion that a steady state of penetration is attained in a

definite time which can be calculated, whereas Longsworth's more rigid method of analysis shows that, strictly speaking, a steady state is not reached in any finite time.

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## BIOLOGICAL OXIDATIONS AND REDUCTIONS\*

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### OXIDATION-REDUCTION POTENTIALS

The number of known, naturally occurring, reversible oxidation-reduction systems has increased considerably within the last year. Especially worthy of mention are the lyochromes and flavins,<sup>1</sup> which are exceptionally widely distributed in the plant and animal worlds. The flavins have the most negative potentials yet encountered among natural oxidation pigments. That of the pigment obtained from Warburg's yellow ferment by exposure to light in alkaline solution amounts to  $-0.208$  v. at pH 7.0 [Bierich and associates (1, 2)]. The values for a lumiflavin from horse liver have been measured by Stern (3) from pH  $-0.2$  to pH 12.4;  $E_h = -0.227$  v. at pH 7.0. Below pH 2.0 oxidation takes place in two stages as detected by the method first applied by Michaelis. Kuhn & Moruzzi (4) have compared the potentials of pure lactoflavin and some of its substitution and degradation products. The potentials with regard to the normal hydrogen electrode at pH 7.0 are: lactoflavin ( $C_{17}H_{20}N_4O_6$ ),  $-0.21$  v.; tetraacetyllactoflavin,  $-0.19$  v.; lumilactoflavin,  $-0.22$  v.; N-monomethyl lactoflavin,  $-0.215$  v.; N, N'-dimethyl lactoflavin,  $-0.20$  v.; a degradation acid ( $C_{12}H_{12}N_2O_8$ ),  $-0.20$  v. The maximum difference is only 30 mv. Recently measured potentials of some plant and animal pigments are given in the following table:

Pigment	Source	E at pH 7	Author
Phoenicin	<i>Penicillium phoeniceum</i>	$+0.047$ v.	Friedheim (5)
	<i>Arion rufus</i>	$+0.025$ v.	Friedheim (6)
Hallachrome	<i>Halla parthenope</i>	$+0.022$ v.	Friedheim (7)
Juglon	Walnut shells	$+0.033$ v.	Friedheim (8)
Lawson	Henna plant	$-0.139$ v.	Friedheim (8)
Phthiocol	Tuberculosis bacillus	$-0.179$ v.	Ball (9)

The question of the reversibility of the oxidation of ascorbic acid has again been investigated, this time by Green (10) who finds that the potential is independent of the concentration of the reactants and

\* Received February 25, 1935.

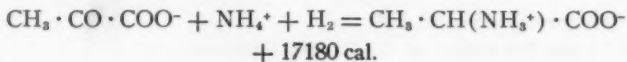
<sup>1</sup> Cf. also this volume, pp. 32, 338, 490. (EDITOR.)

only very slightly affected by the pH value. It is therefore, in his opinion, not reversible in the thermodynamic sense. On the other hand, Fruton (11) claims that it is reversible within the pH range 5.5 to 7.5, the potential having been measured indirectly from the degree of decolorisation of indicators;  $E_h$  at pH 7.0 =  $-0.08$  v. The curves show the theoretical slope, 0.06.

With regard to oxidation and reduction systems set up in the presence of enzymes, the reversibility of Green & Stickland's (12) hydrogenlyase from *B. coli* has been investigated. It proved to be a perfect catalyst for the reaction:  $H_2 \rightleftharpoons 2H^+ - 2e$ , in presence of methylviologen. Measurements at various hydrogen pressures and pH values agreed with the theoretical to within a few millivolts. This system, therefore, behaves equally towards platinum-black or palladium-sol catalysis.

The system hypoxanthine: xanthine: uric acid, in the presence of xanthine oxidase, is also recognised as being reversible. Mlle. Filitti (13) was the first to measure the potential, but her results cannot be considered exact as she did not take the equilibrium, hypoxanthine: xanthine, into account. Green (14) has not left this out of his calculations (oxidation in two stages according to Michaelis) and has arrived at the values,  $E_h$  at pH 7.0 =  $-0.371$  v. for the system hypoxanthine: uric acid, and  $E_h$  =  $-0.361$  v. for xanthine: uric acid, having made colorimetric and potentiometric measurements with methylviologen as indicator. According to the equilibrium constants calculated from these values, xanthine and hypoxanthine must be formed anaerobically from uric acid in detectable amounts, and uric acid ought to be formed from these with the help of xanthine oxidase, which has actually been found to be true.

Measurements of the equilibrium between lactic acid and pyruvic acid have recently been repeated by Wurmser & Mayer-Reich (15) using Stephenson's lactic acid dehydrogenase, and by Barron & Hastings (16) with  $\alpha$ -hydroxidase from gonococci. The former obtained the value  $E_h$  =  $-0.154$  v. at pH 7.0 and 37°, and the latter,  $-0.150$  v. at 35°, which is in good agreement. From this value and the known free energies of formation of water, the ammonium ion, alanine, and lactic acid the former authors have calculated the maximum work for the synthesis of alanine from pyruvic acid (17):



Following Barron & Harrop's (18) observation that the respiration of red blood cells was increased by the addition of methylene blue, similar effects have been noted in other respiratory systems with various reversible indicators. Warburg's explanation that the methylene blue brings about the oxidation of haemoglobin to methaemoglobin is no longer tenable in the light of recent work (19). In the first place, Barron (20) observed that the increase is not sensitive to hydrocyanic acid and, secondly, that other indicators which are not too negative to be able to carry out such an oxidation also intensify the respiration (21). For example, Euler & Adler (22) find that lactoflavin is able to increase the respiration of *thermobacterium helveticum* to as great an extent as methylene blue. Lawson, which cannot form methaemoglobin, causes as large an increase as the more positive juglon [Friedheim (8)]. Pyocyanine produces a twenty-four-fold increase in the respiration of bacteria, and a similar effect on that of various tissues [Friedheim (23)]. De Meio, Kissu & Barron (24) find that a number of indicators which do not form methaemoglobin are still able to intensify the respiration of various tissues. The respiration of kidney-, liver-, and testicle-tissue, on which reversible pigments have no influence, is, however, intensified subsequent to poisoning by carbon monoxide. This shows that the added reversible pigments can take the part of the poisoned natural hydrogen carrier.

#### THE DEHYDROGENASES AND THEIR REACTIONS<sup>2</sup>

When one considers the large number of different dehydrogenating enzymes occurring in nature, one realises how difficult it is to introduce a satisfactory system of classification, especially in a short report like this summarising the work of the last year. Specificity with regard to substrate and acceptor is liable to vary with the source and method of extraction, and it is not always easy to decide whether one is dealing with a single enzyme or a mixture of several. Dehydrogenases named after the same substrate may have very little in common as shown by Boyland & Boyland (25) in the case of lactic acid dehydrogenase from dried yeast and from heart muscle. Using the methylene blue technique, the heart-muscle preparation can dehydrogenate malate, while the other is unable to do so. The optimum pH values are 6.4 for the yeast enzyme and 9.3 for the other. On the other hand xanthine oxidase and succinic acid oxidase are examples

<sup>2</sup> Cf. also this volume, pp. 44, 456, 593. (EDITOR.)



of well-defined, specific enzymes. Warburg & Christian's enzyme (26) from red-cell haemolysate is very specific [Runnström, Lennerstrand & Borei (27)]. It can dehydrogenate hexosemonophosphoric acid and hexosediphosphoric acid aëroically in the presence of methylene blue, but is without action on glycerophosphoric acid, glyceraldehydophosphoric acid, phosphoglyceric acid, methylglyoxal, or dihydroxyacetone. Warburg's coferment accelerates the reaction. The higher-fatty-acid dehydrogenase described below must also be mentioned in this connection.

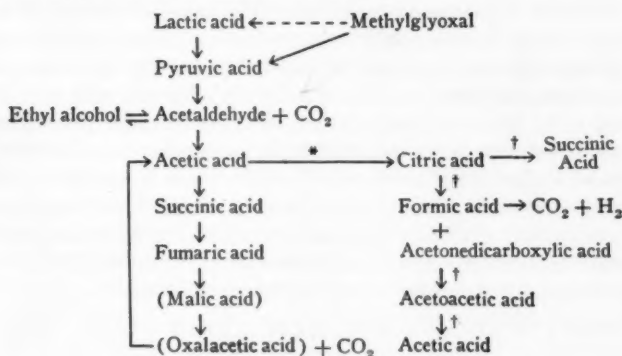
*The dehydrogenating enzymes of yeast.*—It has long been known that extracts are much more specific than the original material, especially in the case of yeast. An acceptor specificity may also appear. Lactic acid dehydrogenase from yeast juice attacks lactic acid when methylene blue or dinitrobenzene, but not oxygen, are the acceptors. Pyruvic acid (15 per cent) and acetaldehyde (10 per cent) were isolated as products (28). A similar development of specificity has been observed by Reichel & Wetzel (31) in the behaviour of liver aldehyde dehydrogenase. Propylaldehyde was only dismutated by a dried preparation under oxygen, whereas dehydrogenation also took place on using methylene blue. An alcohol dehydrogenase, obtained from the juice of ground beer-yeast by precipitation with alcohol and ether, could convert isopropylalcohol to acetone using either oxygen or methylene blue [Müller (29)]. The aërobic reaction appeared quite insensitive to hydrocyanic acid, being only 10 per cent less with 0.01 *M* HCN, and 48 per cent less with 0.1 *M* HCN (30), contrary to the intense decrease in the oxidation when the intact yeast is used. The fact that such a dried preparation takes up only 0.6 to 0.13 cc. per gm. per hour, while "impoverished yeast" [Wieland & Claren (32)], shaken with alcohol, requires 30 to 40 cc., shows how undependable are the conclusions drawn from work on such isolated enzymes, when applied to the entire dehydrogenating system of yeast.

Wieland (cf. 33),<sup>a</sup> in his investigations on the dehydrogenating system of yeast, places particular importance on the quantitative isolation of all the reaction products. Lactic acid, like alcohol, is almost completely oxidised to carbon dioxide. Acetic acid and alcohol are intermediate products, the latter being formed by dismutation of acetaldehyde. Small quantities of pyruvic acid, acetaldehyde, and succinic acid could also be isolated (34). Unlike lactic acid, pyruvic acid

<sup>a</sup> See also Bertho, A., *Ann. Rev. Biochem.*, 3, 27 (1934).



is only partially oxidised to carbon dioxide, reappearing as acetaldehyde, alcohol, and acetic acid. Methylglyoxal, as long as any is left over, is also incompletely oxidised, acetaldehyde being the main product. Other products are pyruvic acid, alcohol, and acetic acid. It has not yet been explained why the intermediate products—in the case of lactic acid, alcohol, and acetic acid—being in an active nascent state, are quickly oxidised, while those in the case of pyruvic acid and methylglyoxal tend to remain. The dismutation of methylglyoxal to lactic acid plays a negligible part in dehydrogenation by "impoverished yeast." The following plan, showing intermediate products isolated, will make clear the results of Wieland's investigations on enzymic oxidation with "impoverished yeast."



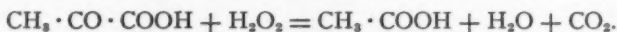
\* In presence of barium ions.

† Anaerobically.

When "impoverished yeast" is used in conjunction with methylene blue, alcohol is dehydrogenated to acetic acid but not further, as the latter is not attacked (35). Lactic acid is also incompletely oxidised (36). In this case acetaldehyde is the main product and not acetic acid, as one might expect from the results of the aërobic experiments. It appears that the energy of the dehydrogenation with methylene blue is insufficient to bring about the rapid series of reactions which oxygen can. Colorimetrically the rate of decolorisation of the methylene blue indicates a reaction of the first order, while the aërobic reaction, being largely independent of the oxygen pressure, is of zero order. A possible explanation is the low rate of diffusion of the methylene blue. This acceptor has also an inhibiting effect on the aërobic reaction, due

probably to displacement from the enzyme surface. The effect amounts to about 50 per cent in the case of acetic acid, which methylene blue cannot dehydrogenate, and only to 30 per cent with alcohol, due to the re-oxidation of leuco methylene blue formed by dehydrogenation. The oxidation of alcohol by yeast poisoned with hydrocyanic acid is accelerated by the addition of methylene blue, since the decolorisation is hardly affected by the poison. The slight poisoning effect which does, however, take place helps to support Wieland's view that the two types of oxidation proceed in essentially the same manner. Similar experiments have been made with quinone as acceptor, which, however, can only be used in concentrations up to 0.01 *M*, since above this value an irreversible deterioration of the yeast commences.

*Bacterial dehydrogenases.*—Davis (37) has obtained acetone preparations from *B. delbrücki* which, unlike the original bacteria, could not ferment glucose, but could still oxidise lactate and pyruvate. With the former, hydrogen peroxide was formed, but not with the latter. Sevag (38) has also made similar observations with pneumococci, whose respiration is not affected by hydrocyanic acid. Correspondingly, as Bertho first showed with *B. delbrücki*, a 50 per cent yield of hydrogen peroxide is obtained when they act aerobically on glucose and lactic acid. With pyruvic acid no hydrogen peroxide resulted. Sevag supposes that the hydrogen peroxide is removed by the following reaction which has been investigated by Hollemann:



The respiratory system of *Azotobacter chroococcum* has been further investigated by Endres (39). Using glucose and alcohol as substrates, the reaction above pH 7 is of zero order. At lower pH values the rate of oxidation of glucose decreases considerably, while the oxidation of alcohol is less sensitive to pH changes. A copper-ion concentration of  $0.6 \times 10^{-4}$  mols. per litre is sufficient to stop the respiration completely and irreversibly, unlike hydrocyanic acid.

For the first time an intermediate product in nitrogen assimilation has been separated (40) and oxidised by iodine to nitrous acid. In all probability the product is hydroxylamine, which is possibly attached as an oxime to the pyruvic acid formed from lactic acid.

Wieland & Sevag (41) find that butyric bacteria are able to dehydrogenate glucose, lactic acid, and various alcohols with oxygen, methylene blue, or quinone as acceptor. The aerobic and anaerobic reactions do not proceed exactly parallel. The bacteria contain cata-

lase and are therefore sensitive to hydrocyanic acid, corresponding to Wieland's conception of the part played by catalase in respiration.

The kinetics of the reduction of methylene blue by glucose, succinic acid, and formic acid in the presence of *B. coli* have received a thorough investigation by Yudkin (42). In the case of succinic acid the rate of reaction falls off rapidly, but slowly increases with the other two. When a second portion of methylene blue is added, it is therefore decolorised rapidly by formic acid and glucose but slowly by succinic acid. Leuco methylene blue has no effect on the times of reductions in all three cases. It may be supposed that the increase in rate is due to the formation of intermediate products, although an active product from formic acid is difficult to imagine.

*Muscle dehydrogenases.*—Yamamoto (43) finds that heart-muscle dehydrogenase in the presence of its coferment is specific for  $\alpha$ -hydroxy acids with methylene blue as acceptor. Apart from lactic, malic, tartaric, and  $\alpha$ -hydroxyglutaric acids, only fumaric acid is attacked, which, of course, changes easily into malic acid. On the other hand, glycollic, mandelic, and phenyllactic acids are not affected. While this dehydrogenase is not optically specific with regard to lactic or malic acids [but cf. Banga, Szent-Györgyi & Vargha (43a)], it dehydrogenates *l*-tartaric and not *d*-tartaric acid. Since lactic acid ester is not dehydrogenated, Yamamoto supposes that the carboxyl group is necessary as an anchor at the enzyme surface, which supposition is supported by the fact that oxalic acid prevents the dehydrogenation of lactic acid supposedly by anchoring itself on to the surface and so displacing the lactic acid. Malonic and glutaric acids also show this effect but in a markedly less degree. Methylene blue, in conjunction with the dehydrogenase of washed pigeon-breast muscle, which also requires Szent-Györgyi's coferment, is most rapidly decolorised by glyceraldehyde phosphoric acid and fructosediphosphate and, somewhat more slowly, by lactic acid [Gözszy (44)]. Succinic acid appears to take the part of hydrogen carrier to this enzyme [Gözszy & Szent-Györgyi (45)]. Since the washed muscle only dehydrogenates lactic acid with methylene blue and not with oxygen, it is probable that a second coferment has been washed out, perhaps succinic acid. In support of this, the fact is cited that succinic acid is always found in muscle, although it should be entirely oxidised away in the presence of oxygen. Fumaric acid is recovered as fumarate (25 per cent) or malate (75 per cent) in dehydrogenation experiments, in which, judging by the oxygen uptake, it ought to have been oxidised to carbon

dioxide. A carrier action of the system fumarate-succinate-succinoxidase, whose oxidation-reduction potential is roughly the same as that of methylene blue, is therefore to be suspected.

*Carrier-linked reactions between isolated dehydrogenases.*—Rather novel reactions between isolated dehydrogenases have been investigated by Green, Stickland & Tarr (46), who brought together in pairs, positive and negative oxidative systems. The dehydrogenases were previously freed from protein and from natural pigments by kieselguhr. The addition of an indicator was found to be necessary in order that the acceptor and donator could react with one another by means of the two dehydrogenases. The following were used as negative systems: formic dehydrogenase and formate, lactic dehydrogenase and lactate, xanthine oxidase and hypoxanthine, succinoxidase and succinate, glucose dehydrogenase (Harrison) and glucose, and the Warburg hexosemonophosphate system; as acceptors: succinoxidase and fumarate, nitratase and nitrate, lactic dehydrogenase and pyruvate. The reactions were followed analytically. It was shown that the added indicator was decolorised by the reducing systems, and again oxidised on addition of the oxidative system. The most active indicators were found to have oxidation-reduction potentials between those of the oxidising and reducing systems, while the activity disappeared outside of these limits. All good indicators were basic in nature; acidic ones did not act well. Pyocyanin was the most suitable. Such hydrogen carriers as glutathione, ascorbic acid, cytochrome-c, and the yellow ferment did not act. The inactivity of the last, when the Warburg system is used as the negative system, is held as evidence against Warburg's view that the yellow ferment manifests its activity mainly in anaërobic systems. The necessary concentration of the dyestuffs is very small. Nile blue and capri blue are capable of action in conjunction with the system formate-nitrate at a concentration of only  $3 \times 10^{-7} M$  at which their colours can no longer be detected. All attempts to isolate a natural carrier from bacteria of various kinds have failed. Perhaps the intact cells do not require such carriers, since the enzymes may lie so close together that they can react directly.

#### THE OXIDATION OF THE FATTY ACIDS<sup>4</sup>

Besides Knoop's well-known  $\beta$ -oxidation of fatty acids, another method by which the animal organism can bring about the degradation

<sup>4</sup> Cf. also this volume, pp. 83, 87, 214, 264. (EDITOR.)

has been lately discovered. This is the oxidation of the terminal methyl groups [Verkade *et al.* (47, 48, 49, 50)]. In experiments to determine whether ketonuria disappeared on feeding only fat obtained from fatty acids with an uneven number of carbon atoms, as should be according to Knoop's theory, a decrease in the amount of ketone bodies in the urine was observed, compared to the controls, when pure triundecylin was given to normal individuals. Considerable amounts of undecane dicarboxylic acid were isolated from the urine. The behaviour of fats built up from one fatty acid on investigation showed that tricaprins was very "diacidogenic," triundecylin even more so, but trionylin and tricaprylin considerably less. Tridecylin was very weakly diacidogenic. This diacidogenic behaviour is therefore not confined to fatty acids with an uneven number of carbon atoms. It commences with the nonylic acids, increases to the undecylic, and is hardly shown by the tridecylic acids.

A double form of  $\beta$ -oxidation of the dibasic acids formed can also take place. On feeding triundecylin to an animal large amounts of undecane dicarboxylic acid, azelaic acid, and pimelic acid appear in the urine. From tricaprins, sebacic acid and small amounts of adipic and suberic acids are formed. Flaschenträger (51) explains this  $\omega$ -oxidation by supposing that the lipases cannot saponify these fats quickly enough and that the esterified acids are not attacked by the dehydrogenases, whose action is therefore confined to the end methyl groups. Besides Yamamoto (43), who used heart-muscle dehydrogenase, Euler (52) has noted that the time of decolorisation for lactic ester is greater than that for the free acid, using yeast dehydrogenase. Flaschenträger fed lauric acid blocked in the  $\alpha$ -position by the benzenesulphonylmethylamino group. Adipic acid, substituted by this group in the  $\alpha$ -position, did not appear in the urine. Since then, however, it has been found that trilaurin itself undergoes  $\omega$ -oxidation. The part played by  $\omega$ -oxidation in animal metabolism cannot be very important, for the dibasic acids formed cannot be utilised and are removed in the urine to the extent of 50 to 70 per cent. Flaschenträger refers to it as an anomalous side reaction.

More is now known about those enzymes which dehydrogenate higher fatty acids. Tangl & Berend (53, 54) have discovered a "desaturase" in the pancreas, which in conjunction with bile can form unsaturated fatty acids from saturated, as recognised by the increase in the iodine number. Quagliariello (55) has noted an increase in the oxygen uptake of bile and fat-tissue cells on addition of the salts of

the higher fatty acids. The investigation of this dehydrogenase by Mazza and associates has shown that, apart from liver and fat-tissue, slices and extracts of such other organs as spleen, pancreas, duodenal mucosa, muscle, and kidney have no oxidative effect on higher fatty acids (56). On the other hand, however, a phosphate extract from liver, from which any donators present had been largely removed by a process of shaking with oxygen, similar to Wieland's impoverishing of yeast, was able to dehydrogenate sodium stearate and palmitate (57). This aerobic reaction is very sensitive to hydrocyanic acid. Methylene blue and quinone could also take the place of oxygen, the rate of reaction increasing in the order: methylene blue, oxygen, quinone. Palmitic methylester was not attacked, in agreement with the opinion already expressed. The first product of the dehydrogenation of stearic acid should be oleic acid, as demanded by the dehydrogenation theory, which, however, is not further oxidised by liver extract (58). The enzyme which converts the  $\alpha$ : $\beta$ -unsaturated acids to the  $\alpha$ -hydroxyacids, according to Knoop's theory, is no longer present in the extract. Liver slices, however, will oxidise oleic acid just as well as stearic acid.

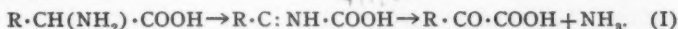
A higher-fatty-acid dehydrogenase has also been observed in *B. coli* [Mazza & Cimmino (59)] for the first time. It is able to dehydrogenate sodium stearate, palmitate, and oleate aerobically, the first decidedly faster than the second. In general, the activity of *B. coli* decreases from formic acid to the acids with six and nine carbon atoms, and then increases again. The rate of the reaction increases in the first hour and then remains steady, representing a state of equilibrium of all the reaction products, as illustrated by the fact that little carbon dioxide is evolved in the first hour. The oxidation of oleic acid begins after an induction period of half an hour. Oxygen may be replaced by methylene blue.

According to Quastel, *B. coli*, after treatment with toluene, can no longer oxidise monosaccharides and lower fatty acids, but still remains active towards higher fatty acids and succinic acid. In this case, however, the reaction velocity decreases slowly with time. Probably the  $\alpha$ : $\beta$ -unsaturated acids remain unattacked. The reaction is brought to a standstill by 0.001 *M* HCN, and reduced 65 per cent by fluoride. The greater sensitivity towards heat shows that this enzyme is different from other dehydrogenases in *B. coli* (60). Unlike other oxidations, that of the higher fatty acids is very sensitive to carbon monoxide, 10 per cent being sufficient to diminish the extent of the

reaction by 90 per cent. Copper does not appear to play any part in this oxidation, since it is not influenced by 8-hydroxyquinolinesulphonic acid or 1-amino-8-naphthol-4-sulphonic acid as is the formic dehydrogenase of *B. coli* (61). Higher-fatty-acid dehydrogenations may therefore be considered as a type apart, and are physiologically of exceptional importance in fat degradation.

#### THE OXIDATION OF AMINO ACIDS<sup>5</sup>

Recent experiments, especially those of Krebs (62), have gone far in supporting Knoop and Neubauer's scheme of amino acid degradation, which was previously based on the results of feeding animals on aromatic amino acids. It was assumed that amino acids do not undergo the chemically improbable hydrolysis, but are oxidised according to the following scheme, which complies with the dehydrogenation theory:



This assumption was supported by the fact that phenylpyruvic acid was found in the urine when phenylalanine was fed. The corresponding keto acids have never been obtained from the ordinary aliphatic amino acids.

Krebs has established, by the systematic study of tissue slices from different organs, that liver and especially kidney are able to deaminate amino acids. He found, however, that ammonia was only eliminated in the presence of air. The keto acids formed (see equation I) are, of course, quickly removed by the tissue, unless an addition of 0.002 *M* HCN or 0.001 *M* As<sub>2</sub>O<sub>3</sub> be made. The enzyme solution from kidney contains an oxido-desaminase and dehydrogenates only as far as the keto acid. In all investigated cases the proportion, used oxygen: ammonia: keto acid, was found to be 1:2:2, as required by the equation. The keto acid could be isolated and identified, thus establishing this mechanism, at least as far as the animal organism is concerned.

Although Krebs could not observe deamination in the presence of methylene blue instead of oxygen, Bernheim & Bernheim (63) have shown that various amino acids can bring about a decrease in the time of decolorisation. They found also that the aerobic oxidation of tyrosine is not hindered by less than 0.005 *M* KCN, while that of phenylalanine is even unaffected by higher concentrations. Krebs also

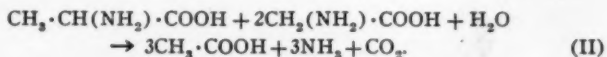
<sup>5</sup> Cf. also this volume, pp. 160, 231. (EDITOR.)



ascribes an optical specificity to the desaminase, which shows a surprising preference for the isomer which does not occur naturally. Neuenschwander-Lemmer & Leövey (64) have attempted to explain this.

The plant organism is also able to degrade amino acids in this manner, according to Grassmann & Bayerle (65) who have tried to explain the mechanism of amine formation in certain plant petals, extracts of which are able to break down amino acids, which are probably the mother substances of the amines present. Oxidative deamination, but not decarboxylation, was observed. It therefore seems likely that the amines are built up by way of the imino acids or the aldehydes.

In the case of *Clostridium sporogenes*, which can live exclusively and anaerobically on amino acids, Stickland (66) has shown that neither hydrolysis nor decarboxylation is the source of the energy, but, strangely enough, the organism can bring about hydrogenation and the opposing dehydrogenation of the amino acids. While the aerobic reaction only proceeds slowly after an induction period of several days [Bessey & King (67)], methylene blue and cresyl violet are rapidly decolorised by alanine, valine, leucine, and phenylalanine, the last acting not quite so fast as the other three. Two mols of cresyl violet are required to one of alanine. Pyruvic acid can also serve as donor, leading finally to the formation of acetic acid. Stickland has observed that in this case *d*-alanine again is the active isomer, the naturally occurring *l*-alanine being scarcely touched. Possible acceptors are glycine, *l*-proline, and *l*-hydroxyproline, the first being dehydrogenated by leucomethylviologen, and partially by benzylviologen corresponding to its oxidation-reduction potential of  $-0.3$  v. (pH 7.0). The potential of proline lies between  $-0.25$  v. and  $0.08$  v. When the two classes of amino acid are allowed to react together in the presence of *Cl. sporogenes*, ammonia is set free. Two mols of glycine are required to dehydrogenate one of alanine, three of ammonia being liberated, probably according to the equation:

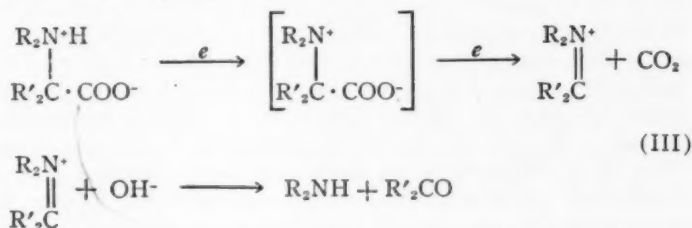


In connection with model experiments of amino acid degradation, mention must be made of Wieland's painstaking proof (68) that the presence of adsorbed oxygen is responsible for the catalytic decomposition of amino acids by animal charcoal. This reaction is represented by equation I. Acetaldehyde, with pyruvic acid as intermediate



product, and equivalent amounts of ammonia and carbon dioxide were isolated. Lactic acid, however, was not formed from alanine, in opposition to the views of the Baur school (69).

Bergel & Bolz (70) have investigated the oxidation of dialkyl-amino acids by animal charcoal, which obviously cannot proceed according to equation I. Trimethylamine is also oxidised to dimethylamine and formaldehyde. They suggest that a "moloxide" is first formed (i.e., one mol of oxygen is attached to one of amine). From experiments with silver oxide, Herbst & Clarke (71) hold that the reaction takes the following course, one atom of hydrogen being removed by dehydrogenation:



More support is lent by the fact that  $\alpha$ -dimethylaminoisobutyric acid is easily oxidised, although all the hydrogen atoms necessary in equation I are blocked. They mention, however, that the oxidation of aminoisobutyric acid in the body has not yet been observed, nor after feeding it has acetone been found in the urine.

#### PASTEUR'S REACTION

Displacement of the anaërobic vital fermentative processes by allowing access of air is generally termed the Pasteur reaction, after its discoverer. Although Meyerhof attributes the decrease in glycolysis under aerobic conditions to resynthesis of glycogen from lactic acid, it is now recognised that such a coupling of the Pasteur reaction and carbohydrate resynthesis is not so important as once believed.

From manometric gas measurements Meyerhof & Lohmann (72) assumed such a far-reaching carbohydrate resynthesis in yeast-oxidation reactions. Experiments on the dehydrogenating enzyme system of yeast by Wieland and his school have, however, not confirmed such an assumption to any great extent, no appreciable resynthesis being found from any of the investigated substrates. After oxidation of lactic acid by "impooverished" beer yeast no increase in the dry weight

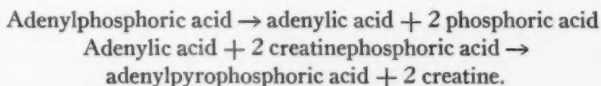
of carbohydrate was found. The entire amount of lactic acid was recovered in the form of its oxidation products [Wieland, Claren & Pramanik (34)]. In the case of alcohol and acetate, Wieland & Wille (35) find that out of 14 mols of the former only one is converted to carbohydrate and two to fat, and only 6 per cent of the latter goes to fat and none to hydrolysable carbohydrate. Resynthesis of carbohydrate and fat from these substances had previously been observed by Smedley-MacLean & Hoffert (73, 74), among others.

The influence of oxygen on yeast fermentation is supposed by Lipmann (75) to be due to a reversible oxidative incapacitating of the necessary catalysts. He attempts to prove this by showing that fermentation by ground yeast juice, itself not sensitive to oxygen, is decreased by the addition of oxidising agents such as iodine, and oxygen in the presence of positive oxidation-reduction indicators. The same applies to glycolysis in muscle extracts. The respiration obtained by the addition of the indicator is not nearly sufficient to attribute the decrease in the anaërobic reaction to resynthesis. From experiments following the influence of the oxidation-reduction potential on the fermentation and respiration of various sorts of yeasts, Kluyver & Hoogerheide (76) have found that, compared with the fairly constant potential of fermenting yeast under oxygen, the addition of an oxidation-reduction indicator increases the potential, the more the respiration of the yeast in question is increased, and the fermentation diminished, by oxygen. This can only be understood if the catalyst promoting the fermentation determines this potential, and if its oxidation reversibly prevents the fermentation. Further evidence in favour has been brought forward by Lipmann (77) who notes that glucose, which causes a lessening of the potential of ground yeast juice under anaërobic conditions, has no influence in the presence of bromoacetic acid. Based on glycogen estimations, Elliott & Schröder (78) have proved that no resynthesis takes place when lactate or pyruvate is aërobically converted by rabbit-kidney cortex. The respiration, glycolysis in Meyerhof's sense, and the Pasteur reaction are not interconnected in the intestinal mucous membrane of the guinea pig [Bumm, Appel & Fehrenbach (79)], a considerable increase in glycolysis taking place at an oxygen partial pressure of 10 per cent as compared with pure oxygen, whereas no decrease in the respiration was noticeable. All these latest observations support the conclusion that it is no longer admissible to estimate the degree of resynthesis from the extent of the Pasteur reaction in fermentation and glycolysis.

## THE PROBLEM OF COFERMENTS\*

In the determination of the molecular weight of cozymase by diffusion methods, Myrbäck has obtained values between 450 and 500 (80). Since the value for adenylic acid is only 347, it would appear that this is not the only constituent. The highest "covalue" yet measured is 179,000 after purification. The purest preparations have a nitrogen content of 15 per cent, of which 80 to 85 per cent is due to purines, which points to the participation of other nitrogen compounds. Cozymase in solution acts as a monobasic acid on titration, but on heating with dilute alkali another acid group is set free, the activity being lost at the same time. The constitution is therefore best represented at the present time by the following: adenosine —  $\text{PO}(\text{OH}) - \text{R}$ , where R probably contains an atom of nitrogen and is phosphorus-free.

Lohmann (81) maintains that the coferment for the formation of lactic acid is adenylypyrophosphoric acid, being at the same time also the coferment required in the cleavage of creatinephosphoric acid, which is the other reaction supplying the energy necessary for muscle contraction. The part played by the adenylyphosphoric acid is as follows (82):



Some light has been thrown on the complicated systems of coferments at work in the various dehydrogenations by Andersson (83). He claims to have established that the coferment from the boiled juice of pigs' hearts [Banga & Szent-Györgyi (84)] is identical with cozymase, by comparing their activities towards fermentation and dehydrogenation [but cf. Birch & Mann (85)]. This is very probable in the case of the glucose dehydrogenating coferment from *B. coli* discovered by Harrison (86). Yudkin (87) has also come to this conclusion. It is also active towards hydrogenlyase. Formate dehydrogenase from peas and alcohol dehydrogenase from dried yeast also require cozymase, but muscle succinodehydrogenase does not require any coenzyme. Holmberg (88) has shown that cozymase is the coferment in the dehydrogenation of hexosediphosphate by washed muscle.

Warburg & Christian (89) have proved that the coferment from horse blood cells is not the same as cozymase, although probably very

\* Cf. also this volume, pp. 48, 177. (EDITOR.)

similar judging from analyses of highly pure preparations. Acid hydrolysis gave 70 per cent of adenine nitrogen. Two bases of a nature as yet unknown have also been isolated, one being found to be identical with nicotinic amide.

#### WARBURG'S YELLOW FERMENT AND THE FLAVINS<sup>†</sup>

The yellow ferment has been further purified by Theorell (90) by cataphoresis till it finally has a content of 0.44 per cent coloring matter calculated as lactoflavin. This can be separated from the protein by dialysis in hydrochloric acid solution. On mixing the two components, which are themselves inactive, the greater bulk of the activity can be recovered (91). The protein, however, could not be activated by the addition of pure natural lactoflavin or Kuhn & Weygand's (92) synthetic 6,7-dimethyl-9-*l*-araboflavin which can be explained by the fact that the coloring matter obtained after precipitating the protein with methyl alcohol contains exactly one atom of phosphorus per molecule and is probably a lactoflavin phosphoric acid. This agrees with the results of cataphoresis; the coloring matter travels to the anode with the approximate velocity of a phosphoric ester, unlike lactoflavin, which does not migrate at all.

Warburg & Christian (93) built up their system from the yellow ferment, *Zwischenferment*, from yeast, and cozymase from red blood-cells, but Euler & Adler (94) have shown that the yellow ferment may also function in other systems. The dehydrogenase obtained from apozymase by precipitation with acetone is free from cozymase and flavin, and dehydrogenates alcohol and the Robison ester aerobically or with methylene blue, only after the addition of flavin enzyme and cozymase. Such a coöperation of three components can also be demonstrated by using Warburg's *Zwischenferment* and cozymase with alcohol and the Robison ester. On the other hand, using Warburg's system, Bertho & Zychlinski (95) could only dehydrogenate the Robison ester and not other donators such as glucose. The source of this discrepancy probably lies in the preparation of the *Zwischenferment*, which may be regarded as the dehydrogenase really responsible for the activation of the substrate. A dehydrogenase from frog muscle which contained remarkably little flavin was only able to oxidise malic acid in the presence of methylene blue after the addition of the yellow ferment and cozymase [Wagner-Jauregg *et al.* (96)]. Methylene blue could

<sup>†</sup> Cf. also this volume, pp. 17, 338, 490. (EDITOR.)

only be partly replaced by oxygen. The presence of preventive substances in the frog muscle may explain this, since oxygen no longer served as an acceptor after the extract had been added to the *Zwischenferment*.

The flavins, which form the prosthetic group in the yellow ferment, can be used as hydrogen acceptors due to their oxidation-reduction properties. Flavin appears to be most easily decolorised by minced liver after addition of succinic acid [Wagner-Jauregg *et al.* (97)]. This is difficult to understand because succinic acid cannot be dehydrogenated to fumaric acid in measurable amounts by flavin owing to the unfavourable potential values. Although the muscles of cold-blooded animals contain succinic dehydrogenase they cannot decolorise flavin on addition of succinic acid. Only after addition of liver extract, which is in itself inactive, did decolorisation result. It is not yet certain whether this is due to the presence of a positive oxidation system in the liver.

It is not yet known with certainty if the flavin enzyme really is of great importance as a hydrogen carrier in natural oxidations. That the activity of xanthine oxidase is not dependent on the flavin content has been clearly demonstrated by Green & Dixon (98). It must be pointed out, however, that the activity of the above-mentioned enzyme systems is considerably smaller than that of intact yeast cells or that of acetic bacteria, and caution must be exercised before coming to definite conclusions. For instance, Bertho & Zychlinski (95) found that the autolysate after repeated autolysis of lactic bacteria with liquid air was only weakly active, but the residue very much more so. Yet much can be said in favour of the view that the yellow ferment does take part in many oxidations which are not sensitive to hydrocyanic acid. It can be placed side by side with Keilin's system for respiration sensitive to hydrocyanic acid: substrate + dehydrogenase + cytochrome + indophenol oxidase. Experiments corresponding to those reported above, but in the field of cyanide-sensitive systems, have already been made in previous years by Keilin (99) and Harrison (100).

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## ENZYMES\*

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The most notable development in the field of enzymes during recent years has been the isolation of six enzymes and two zymogens in the crystalline state. One now finds very few papers describing attempts to purify or isolate enzymes by methods of adsorption. The discovery that certain, if not all, enzymes are proteins has identified enzyme chemistry with protein chemistry and promises to do much toward clearing up some of the most puzzling facts regarding the high molecular state of proteins.

### CRYSTALLINE ENZYMES

*Crystalline urease.*—The argument as to whether the octahedral crystalline globulin, isolated from jack bean meal by Sumner (1) in 1926, is identical with urease appears to be settled definitely in the affirmative. The claim of Waldschmidt-Leitz & Steigerwaldt (2) that they could digest crystalline urease-protein by trypsin without inactivating the urease has been satisfactorily disproved by Sumner (3) and his associates, who find that even traces of urease continue to give a precipitate with antiurease after being incubated with trypsin-kinase for 48 hours at 37° C. Sumner, Kirk & Howell have shown that at pH 4.3 crystalline urease is rapidly inactivated and digested by pepsin and that the inactivation rate is parallel to the rate of digestion. Waldschmidt-Leitz's (4) reply that this action of pepsin is the result of more complete hydrolysis than is brought about by trypsin appears to the author to be unconvincing, inasmuch as pepsin is known to hydrolyse proteins much less completely than trypsin-kinase. The claim made by Waldschmidt-Leitz & Steigerwaldt (2) that the precipitate given when antiurease is added to solutions of crystalline urease, which have been incubated with trypsin, is composed of urease proper and does not contain the urease-protein has been shown to be untrue by Kirk & Sumner (5). The latter have found that highly purified antiurease, produced by injecting jack bean urease into rabbits, precipitates crystalline urease stoichiometrically and that the antiurease carries down the urease activity at the same rate as it carries down the urease-protein.

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Kubowitz & Haas (6) have found that crystalline urease absorbs light only in the ultra-violet. Using a solution containing but 0.000025 per cent urease, they noted that urease is destroyed by light of exactly those wave-lengths which urease absorbs.

Fankuchen (7) irradiated crystalline urease and crystalline pepsin with X-rays, using the powder method. The spacings for urease in Ångström units were: 11.2, 4.57, 4.22, 4.15, 3.75, 2.34, and 2.13. For pepsin: 49.2, 29.2, 21.0, 15.8, 12.8, 10.7, 9.65, 8.40, 7.16, 4.20, and 2.14. The 4.20 spacing in pepsin was very intense. The results with pepsin do not agree closely with those of Astbury & Lomax (8).

*Crystalline pepsin.*—Northrop's crystalline pepsin has been claimed not to be identical with pepsin proper, both by Waldschmidt-Leitz & Kofranyi (9) and by Dyckerhoff & Tewes (10). The former workers repeatedly adsorbed pepsin upon suspended crystals of cantaloupe-seed globulin. After the peptic activity had been entirely removed from solution they determined the dry matter left in solution and decided that this represented the pepsin protein. They accordingly claimed to have succeeded in separating crystalline pepsin into two components, the active part which was adsorbed upon the cantaloupe-globulin crystals, and the so-called carrier, which was left in solution.

Dyckerhoff & Tewes adsorbed crystalline pepsin upon suspensions of edestin crystals and upon other proteins. They found that the adsorption complexes had a greater peptic activity than the original crystalline pepsin. By allowing crystalline pepsin to autolyse they obtained spheroids which they claimed to possess more peptic activity than the original crystalline pepsin. The work has been repeated by Northrop (11), who found it to be erroneous. He has shown that pepsin adsorbed to cantaloupe-seed globulin, edestin, etc. possesses a peptic activity depending upon its content of crystalline pepsin. He has been able to extract crystalline pepsin from the adsorption complexes and from the spheroid material with 0.25 *N* sulfuric acid and to obtain from these extracts pepsin crystals with characteristic activity.

Sumner (12) also has shown the work of Waldschmidt-Leitz & Kofranyi to be erroneous. He adsorbed crystalline pepsin from solution by shaking with either casein or denatured ovalbumin suspensions at pH 4.8 and found that the peptic activity was adsorbed at the same rate as the pepsin protein. Sumner employed a characteristic test for pepsin-protein, which he determined turbidimetrically after denaturing by alkali and precipitating by addition of acid.

Herriott & Northrop (13) have acetylated crystalline pepsin, using ketene at pH 4.0 to 5.5. Three acetyl derivatives have been isolated in crystalline form similar to that of crystalline pepsin. A compound containing three or four acetyl groups and none of the original amino groups had the same proteolytic activity as the original pepsin. A compound containing six to eleven acetyl groups had about 60 per cent of the original activity. A compound containing twenty to thirty acetyl groups had only about 10 per cent of the original activity.

The solubility, amino "N," acetyl content, isoelectric points, and specific gravities of the acetyl derivatives were significantly different from those of crystalline pepsin, while the pH activity, acid and alkali inactivation and titration curves were not.

J. St. L. Philpot and I.-B. Eriksson-Quesnel (14) have shown, using the ultracentrifuge, that crystalline pepsin solutions contain particles all of which are of the same size. The molecular weight is 35,500.

Gates (15) has found that the absorption spectrum for crystalline pepsin agrees essentially with the destruction spectrum and that it is similar to that found previously for crystalline urease.

The temperature coefficient of inactivation of crystalline pepsin by ultra-violet light is 1.02 (16).

Northrop (17) finds that the loss of activity occasioned by exposing solutions of crystalline pepsin to radium gamma rays and to ultra-violet light is parallel to the decrease in pepsin protein. The rate of inactivation by ultra-violet light depends upon the pH of the solution and is at a maximum at about pH 2.0.

Bernal & Crowfoot (18) have taken X-ray photographs of crystals of pepsin 2 mm. in length in liquid contained in Lindemann glass. The dimensions of the unit cell are found to have a minimum value for  $a$  of 67 Å and for  $c$  of 154 Å. Using the powder method Astbury & Lomax (8) find the chief rings for crystalline pepsin to be 11.5 Å and 4.6 Å.

*Crystalline trypsin.*—Kunitz & Northrop (19) state that crude trypsin is permanently inactivated above 70° C. but crystalline trypsin may be heated to boiling in acid and regain its activity on cooling. Trypsin may be inactivated reversibly or irreversibly. Reversible inactivation is accompanied by the formation of reversibly denatured protein. This is in equilibrium with native active protein. The equilibrium is shifted toward the denatured form by increasing the temperature, or by making the solution more alkaline. On standing, reversibly

denatured trypsin changes into irreversibly denatured trypsin. Trypsin protein is slowly hydrolysed from pH 2 to 9. The point of maximum stability for trypsin is at about pH 2.3.

*Crystalline chymotrypsinogen and chymotrypsin.*—Kunitz & Northrop (20) announce the isolation of a new protein from the pancreas. It separates as elongated prisms and has no proteolytic action. However, when acted upon by a trace of active trypsin it is converted into a second protein, crystallizing in plates, which has a proteolytic activity about a third as great as crystalline trypsin-1. The new enzyme has less hydrolytic action upon gelatin than trypsin-1, but has a powerful action in coagulating milk. In this connection it is of interest to recall that in 1913 Vernon (21) claimed to be able to activate trypsinogen more readily by adding trypsin than by adding enterokinase.

Ten recrystallizations of the elongated prisms caused no change in optical activity. Three recrystallizations of the plates caused no change in the proteolytic activity.

The activation of chymotrypsinogen by active trypsin is monomolecular (22) and is proportional to trypsin concentration. Although chymotrypsinogen cannot be activated by enterokinase, the mother liquor can be activated by half-saturated magnesium or ammonium sulfate at pH 7.0 and 30° C. This reaction is autocatalytic.

Ten Broeck (23) has found that crystalline trypsin from the cow and the pig, as well as chymotrypsinogen and chymotrypsin from the cow, can be differentiated by the anaphylactic test, using rabbits. The enzymes were recrystallized at least five times. Rabbits receiving trypsin and chymotrypsin showed necrotic areas at the sites of injection and some died.

*Crystalline trypsinogen.*—Kunitz & Northrop (24) describe a method for the isolation of trypsinogen from the mother liquor from chymotrypsin. The trypsinogen is obtained as short triangular prisms and is a protein. Upon standing at pH 7 to 8 in concentrated magnesium sulfate it is converted into active trypsin, which can be crystallized in the form of short rectangular prisms or fine needles and is identical with the crystalline trypsin previously described.

The activation curve for crude trypsinogen shows a lag period, due probably to the presence of some inhibitory substance. For crystallized trypsinogen, however, there is no lag period and therefore it is impossible to recrystallize trypsinogen without converting it into active trypsin.

*Crystalline yellow oxidation enzyme.*<sup>1</sup>—Theorell (25) has purified the yellow oxidation enzyme of Warburg & Christian by cataphoresis and by fractionation with ammonium sulfate at pH 5.2. By dialysis against two volumes of saturated ammonium sulfate a crystalline product was obtained. Theorell regards this as a pure substance. The material has a pigment content of 0.45 to 0.47 per cent. The nitrogen content is 15.5 per cent. The colloidal component is a protein.

When the salt-free enzyme is dialysed against dilute hydrochloric acid it is split into its pigment and its protein. The protein is soluble in distilled water, but is precipitated by salts. The two components are entirely inactive but when brought together the activity of the enzyme is largely restored.

*The carrier theory.*—The isolation of enzymes in crystalline form has made it appear very unlikely that there is any such thing as an enzyme carrier and this theory has been attacked by Sumner (26). Sumner points out that hemoglobin cannot be considered as a model representing an active group (hematin) united with a carrier (globin) (27), since hemoglobin is a true chemical compound, the chemical and physical properties of which depend upon the integrity of the molecule as a whole. Likewise, catalase and peroxidase, if they are composed of hematin and some protein, are chemical compounds which lose their enzymic activity almost entirely when the hematin is split off.

#### PROTEOLYTIC ENZYMES

*Pepsin.*—Krijgsman (28) describes a micro-method for the estimation of pepsin. After incubation, the serum protein, edestin, or casein remaining undigested is precipitated by sulfosalicylic acid and is determined nephelometrically.

*Trypsin.*—Kleiner & Tauber (29) allowed pig pancreas to autolyse in dilute alcohol for 18 months. The enzyme preparation, after dialysis and precipitation with alcohol, gave a negative biuret test. Much enzyme was lost upon dialysis.

Anson & Mirsky (30) describe the equilibrium between active native trypsin and inactive denatured trypsin. In a solution sufficiently alkaline the renaturation of trypsin is prevented by urea.

The tryptic digestion of casein and gelatin has been followed by Sreenivasaya, Sastri & Seerangachar (31) in the two-bulbed dilatometer. In early stages changes are registered by the dilatometer, but are

<sup>1</sup> Cf. also this volume, pp. 17, 32, 338, 490. (EDITOR.)

not indicated by increase of amino nitrogen. After this the dilatometer depression is proportional to formation of amino nitrogen. The dilatometric depression per millimol release of nitrogen is 8.7 mm. with gelatin and 10.8 with casein.

*Fig-tree protease*.—Robbins & Lamson (32) find that the trypsin-like enzyme from the sap of the fig tree, which digests live *Ascaris* worms, is present in greatest concentration in *Ficus carica* from Alabama. Considerable amounts of enzyme were found in the sap of six South American species of fig tree. It is of interest that while the *Ascaris* worm is protected by its antienzymes against digestion by pepsin and trypsin it is not resistant to digestion by fig-tree protease.

*Cathepsin*.—Blagowestschenski & Korman (33) have tested the effects of oxidants and reductants upon the point of equilibrium of the cathepsin of *Vicia sativa* when acting upon globulin of sunflower seeds. Contrary to Rondoni & Pozzi, cysteine inhibited hydrolysis. Oxygen also inhibited the hydrolysis as was noted by Laquer and by Waldschmidt-Leitz & Purr. However, a favoring of synthetic action of oxygen could not be demonstrated. It would appear to the author that if the catalyst were affected by the oxidation potential so that the equilibrium of a proteolytic reaction is shifted, then the definition of catalysis would require a drastic alteration.

It has been found by Euler, Karrer & Zehender (34) that the activating action of ascorbic acid on cathepsin is increased by adding ferrous sulfate, ferric chloride, or calcium acetate. Cupric salts inhibit both in the presence and absence of ascorbic acid. Metallic ions do not increase the activating effect of reductone and reductin acid on cathepsin. Besides cysteine, cyanide, and ascorbic acid, hexose phosphate also activates cathepsin.

*Protaminase*.—Weil (35) describes the preparation of protaminase free from other proteolytic enzymes and describes a quantitative method for its estimation. Protaminase is activated by enterokinase and its substrate requires no amino or carboxyl groups.

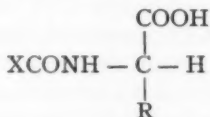
*Papain*.—Hellerman & Perkins (36) think it possible that papain activation may be due to a reduction of  $-SS-$  groups in the enzyme molecule to  $-SH$ . Catalysed oxygen inactivates papain, as do also iodine, quinone, and ferricyanide. Cuprous oxide and mercurials of the type  $RHgX$ , which are known to unite with thiol compounds, completely inactivate papain. The activation of papain by hydrocyanic acid and cysteine suggests a stoichiometric relationship.

Svedberg & Eriksson (37) find that when ovalbumin is digested by



activated papain, non-centrifugable substances, a centrifugable substance about as complex as protamine, and a substance which has about the same molecular weight as ovalbumin, but which has a high dissymmetry, are formed. Unactivated papain had no influence upon the sedimentation constant of ovalbumin and non-centrifugable products were not formed.

*Carboxypolypeptidase.*—Bergmann, Zervas & Schleich (38) find that peptide hydrogen is necessary for action of carboxypolypeptidase, since chloracetyl-N-methyl-L-tyrosine is not attacked. The  $\alpha$ -hydrogen is not necessary, since the enzyme will split pyruvyl-D-L-phenylalanine. For action of carboxypolypeptidase the substrate must have the following atom groups and configuration:



*Aminopolypeptidase.*—Grassmann, Emden & Schneller (39) describe the purification of aminopolypeptidase of yeast, using papain to liberate the aminopolypeptidase.

*Dipeptidase.*—Bergmann & Zervas (40) find that to be split by dipeptidase a dipeptide must contain natural amino acids, a normal peptide linkage, a free carboxyl attached to an immediately adjacent carbon atom, and an amino group likewise attached. The nitrogen of the peptide linkage must have a hydrogen atom. The dipeptide must contain at least one hydrogen on the  $\alpha$  carbon atom and one on the  $\alpha'$  carbon atom. The  $\alpha$  and  $\alpha'$  carbon atoms must have the proper configuration. The protein tie must be in the imide form.

Grassmann & Schneider (41) find that dipeptidase from yeast and from glycerol extracts of intestinal epithelium and kidney split *L*-asparagyl- $\alpha$ -glycine, *L*-asparagyl-diglycine, and *L*-glutamyl- $\alpha$ -glycine, while *L*-asparagyl- $\beta$ -glycine is not attacked. The *D*-*L*-asparagyl-monoglycine of E. Fischer is not attacked and is therefore a  $\beta$  compound.

Linderstrøm-Lang (42) has observed that the splitting of alanyl glycine by intestinal dipeptidase is aided by low concentrations of zinc acetate and is inhibited by higher concentrations. Hydrocyanic acid inhibits in all concentrations.

Buadze (43) finds that normal urine of man, rabbit, and dog contains dipeptidase and polypeptidase. He describes a method for the determination of these enzymes.

*Rennet or chymosin.*—Tauber & Kleiner (44) find that rennet is rapidly inactivated by pepsin at pH 2.3. It is also inactivated by trypsin at pH 6.2. There is no exchange of the so-called protein carrier when rennet is adsorbed upon crystalline edestin.

Lundsteen (45) has employed a method to investigate the purely enzymatic phase of rennet action upon milk and finds the reaction optimum to lie at about pH 5.4.

Sørensen, Holter & Andersen (46) point out pitfalls in work which seeks to differentiate between pepsin and chymosin and note that the peculiarities of the clotting process must be considered in making enzyme determinations which depend upon the observation of this process. The results reported by Menck-Thygesen, Ege & Lundsteen and Kleiner & Tauber are criticized.

*Prochymosin.*—Ege & Lundsteen (47) find that aqueous extracts of calf mucosa at pH 6 contain only 5 to 10 per cent of the active enzyme. At 40° C. the activation begins at a pH below 5. At pH 3.4 activation requires 30 minutes. At pH 3 it requires 1.5 minutes. Upon standing for a long time at pH 7 a spontaneous activation occurs, but this may be due to bacteria.

*Thrombin.*—Strughold & Wohlsch (48) find that trypsin-kinase does not accelerate blood coagulation but inhibits it or stops it completely under suitable conditions. A hydrolytic breaking down of fibrin in blood coagulation is not demonstrable. The hypothesis of Waldschmidt-Leitz that thrombin is a proteolytic enzyme related to trypsin is claimed to be untenable.

#### OXIDIZING AND FERMENTATION ENZYMES<sup>2</sup>

*Peroxidase.*—Elliott & Keilin (49), in opposition to the claim of Kuhn, Hand & Florkin (50) that horse-radish peroxidase is a hematin compound, state that in horse-radish preparations all of the hematin is present in the free state as acid hematin. The nitrogen compounds present do not combine with the hematin in solutions more acid than pH 9. Complete combination occurs only at pH 10.5 and at this pH the peroxidase is nearly inactive. These results may have been due to the fact that the peroxidase preparations of Elliott & Keilin were much less active than those of Kuhn, Hand & Florkin.

*Catalase.*—Carbon monoxide in the presence of nitrogen specifically inhibits catalase (51). The compound CO-catalase is most readily dissociated by light of wave-length 405 mμ.

<sup>2</sup> Cf. also this volume, pp. 19, 456, 593. (EDITOR.)

Sevag & Maiweg (52) find that oximes inhibit catalase if the oxime is previously treated with hydrochloric acid and subsequently neutralized. Unless the oxime is treated in this manner there is no inhibition. Diacetyl dioximes and phenyl glyoxime inhibit catalase more strongly than cyanide at 3° and at 37° C. The inhibition with diacetyl dioxime is completely irreversible.

Remesow (53) finds that colloidal cholesterol decomposes hydrogen peroxide catalytically.

*Uricase*.—Truszkowski (54) has investigated the method of Rô (55) for obtaining soluble uricase and describes a simplified method. *In vivo*, uricase acts as a contact catalyst. Uricase cannot be extracted from fresh tissues on account of the presence of lipids.

*Fumarase*.—Jacobsohn (56) finds that neither diethyl fumarate nor potassium ethyl fumarate is attacked by liver fumarase.

*Xanthine oxidase*.—Green & Dixon (57) find that the ability of xanthine oxidase of milk to react with molecular oxygen is not dependent upon presence of lactoflavin. In the system—xanthine oxidase + hypoxanthine—the oxygen probably reacts directly with the substrate.

Green (58) has demonstrated the reversibility of the xanthine oxidase system, using colorimetric and potentiometric methods. At pH 7.0 the  $E_o'$  of the hypoxanthine-xanthine is -0.371 volt. The  $E_o'$  of the xanthine-uric acid is -0.361 volt. A formation of uric acid and hypoxanthine from xanthine and also a formation of xanthine from hypoxanthine and uric acid have been observed under anaërobic conditions.

*Aldehydease*.—Reichel & Wetzel (59) find, contrary to previous opinion, that aldehydease purely dismutates aldehydes in the presence of oxygen as well as under anaërobic conditions. In the presence of quinone or methylene blue it can dehydrogenate aldehydes.

*Alcohol dehydrogenase*.—Alcohol dehydrogenase of the liver changes alcohol to aldehyde only in the absence of oxygen according to Reichel & Köhle (60). Hydrocyanic acid does not inhibit and methylene blue and quinone increase the yield of aldehyde. Alcohol dehydrogenase of yeast is found by Müller (61) to be 50 per cent destroyed by heating at pH 6.3 at 58° C. for a half hour. The pH optimum is between 7.5 and 10. The activity is little affected by 0.01 M HCN but is decreased one-half by 0.1 M HCN. The precipitated maceration juice contained no succinic dehydrogenase, triose dehydrogenase, or xanthine dehydrogenase.

Lehmann (62) describes a method for the preparation of alcohol

dehydrogenase solutions from Lebedew dry yeast. The enzyme is activated by adenosine triphosphate prepared according to Fiske; otherwise it is inactive. Since oxygen cannot be used as acceptor for this dehydrogenation, the enzyme is classified as an "anoxytropic dehydrogenase."

The alcohol dehydrogenase from muscle, liver, and tumor tissues also requires adenosine triphosphate as coenzyme. It is extremely unstable (63).

Euler & Adler (64) find that alcohol dehydrogenase (65) dialysed free from cozymase can rapidly dehydrogenate ethyl alcohol if purified cozymase and the yellow oxidation enzyme are added. Apozymase as a source of alcohol dehydrogenase can also be employed. Methylene blue, lactoflavin, or atmospheric oxygen can be used as acceptor for hydrogen. The reaction is not sensitive to hydrocyanic acid.

Similar results are obtained using hexose monophosphate as substrate. The authors describe the preparation from apozymase or from Lebedew yeast juice of an alcohol dehydrogenase which is largely free from the yellow oxidation enzyme.

*Lactic dehydrogenase.*—Birch & Mann (66) prepared cell-free lactic dehydrogenase from heart muscle. They find that two factors are necessary for the activation of this enzyme and these can be obtained from heart or yeast. Neither factor is identical with cozymase. The coenzymes have been obtained free from vitamin B<sub>1</sub>.

Barron & Hastings (67) find that when lactate, pyruvate, and a reversible dye are added to  $\alpha$ -hydroxy oxidase at 35° C. a stable potential is attained after 1 to 2 hours. The enzyme was obtained from autolysed gonococci and the dye was a mixture of pyocyanine and cresyl violet. The results obtained by using various ratios of lactate to pyruvate indicate a reversibility of the system. The oxidation-reduction potential was measured at pH values ranging from 5.83 to 7.79. At 35° C. the normal potential of the system was +0.248 volt. The free energy change was calculated to be 11,440 cal. and the heat of the reaction 21,639 cal.

*Glycolytic enzyme system.*—Michaelis & Runnström (68) find that the inactivation of the glycolytic system prepared according to Meyerhof (69) is probably due to oxidation, since the system is reactivated by the addition of thioglycollic acid. They postulate that the glycolytic enzyme is a sulfhydryl compound which is readily oxidized to the disulfide state. This is an interesting development since crystalline urease has been shown to act like a sulfhydryl compound (70)

and since the possibility exists that papain, cathepsin, and arginase also may be sulfhydryl compounds.

*Hydrogenase*.—The enzyme hydrogenase has been studied by Green & Stickland (71) using *Bact. coli* and has been shown to catalyze reversibly the reaction:  $H_2 \rightleftharpoons 2H^+ + 2e$ . This is the most negative oxidation-reduction system thus far observed in living cells.

*Glyoxalase*.—Platt & Schroeder describe a manometric method for glyoxalase (72). At 25° C. and with a low concentration of methyl glyoxal and glutathione the rate of action is independent of methyl glyoxal. With higher glutathione concentration the rate is dependent upon the concentration of methyl glyoxal. Evidence is submitted to support the idea that the real substrate is the glutathione-methyl-glyoxal compound, as believed by Jowett & Quastel (73). Iodoacetic acid inhibits glyoxalase by destroying the glutathione.

The kinetic behavior of animal glyoxalase is shown by Platt & Schroeder (74) to be identical with that of yeast glyoxalase. No relationship was found between the high glycolytic activity of cancer tissue and its glyoxalase content. The kidney is believed to contain an antiglyoxalase.

Jowett & Quastel (75) have investigated glyoxalase in thin slices of tissues. The glyoxalase activity of tissues is greater than hitherto supposed. The diffusion of methyl glyoxal into the slices limits the rate of the reaction. Oxygen partly inhibits glyoxalase activity.

The view of Ochoa & Dudley (unpublished) that the antiglyoxalase effect of pancreatic tissue is due to amino acids and especially to the amino acid histidine is confirmed by Girsavicius, Efendi & Ryzhova (76).

*Thiosulfate oxidase*.—Pirie (77) has found that rat liver, kidney and chorion and goose kidney oxidize thiosulfate to sulfate *in vitro*. There is no perceptible utilization of oxygen; nevertheless the reaction will not proceed anaerobically. Anaerobic incubation causes irreversible inactivation of the agent causing oxidation.

*Respiration ferment*.—Negelein & Gerischer (78) claim that in azobacter, as is also true of acetic bacteria, the oxygen-transporting ferment may be observed directly by the spectroscope. The reduced ferment gives a band at 632 m $\mu$  and, when oxidized, at 647 m $\mu$ .

Keilin (79) finds that all absorption bands of hematin compounds seen by direct spectroscopic examination of cells of different organisms belong either to free hematin or to cytochromes and no band thus far can be ascribed to the oxidase or oxygen-transporting enzyme.

Warburg & Negelein (80) deny Keilin's assertion that the bands in bacteria are due to cytochromes, or cytochrome derivatives.

*Carboxylase*.—Langenbeck, Wrede & Schlockermann (81) describe the purification of yeast carboxylase. The coenzyme plays a less important rôle the purer the carboxylase preparation. The authors are inclined to the view that carboxylase is identical with the carboligase of Neuberg, but have not been able to prove this.

Langenbeck, Hellrung & Jüttemann (82) have studied organic compounds which catalytically decarboxylate keto acids. One compound, 6-hydroxy-3-amino- $\alpha$ -naphthoxy-indol, was found to be 4,000 times more effective than methylamine and to decarboxylate at 37° C. only 22 times less rapidly than crystalline pepsin digests protein at 35.5° C.

*Carboligase*.—Stepanow & Kusin (83) have determined carboligase in the limb muscles and liver of the dog and have found much enzyme in muscle but only traces in the liver. Animal voluntary muscle contains no less than plants. The amount extractable from yeast and green plants is greater than the amount extractable from muscle. The method employs pyruvic acid, which is converted by the enzyme into acetyl methyl carbinol. Carboligase is believed to play an essential rôle in metabolism.

Stepanow & Kusin (84) tried to show that a brew of dog's leg muscles can synthesize glycogen from keto-hydroxy-succinic acid,  $\text{HOOC} \cdot \text{CHOH} \cdot \text{CO} \cdot \text{COOH}$ . The tests showed twice as much glycogen as the controls and contained more monosaccharide also.

*Cozymase*.<sup>3</sup>—Cozymase of yeast is found by Anderson (85) to be identical with the cozymase of lactic acid dehydrogenase and apparently identical with the cozymase of glucose dehydrogenase. The enzymic oxidation of ethyl alcohol and of lactic acid, using dried top yeast or washed heart muscle, is activated by cozymase. Succinic dehydrogenase is not activated by cozymase.

Myrbäck finds (86) the molecular weight of purified cozymase to be at least 450. Either cozymase has nothing to do with adenylic acid, or else it is a compound of adenylic acid or some other substance of unknown nature. A new method of preparing cozymase has been worked out and the cozymase has been shown to contain 80 to 85 per cent of its nitrogen as purine nitrogen (87). The residue combined with the adenine probably contains one atom of nitrogen.

<sup>3</sup> Cf. also this volume, pp. 31, 177. (EDITOR.)

Purified yeast cozymase behaves like a monobasic acid upon titration. After inactivation by heating at neutral or slightly alkaline reaction it acts like a dibasic acid. Myrbäck (88) believes the compound to be:  $C_5H_4N_5 \cdot C_6H_8O_5 \cdot O \cdot PO(OH)NHR$ .

Warburg & Christian (89) have greatly purified the coenzyme of horse erythrocytes. The purified material contains organic esters of phosphorus. Seventy per cent of the total nitrogen is adenine nitrogen. Two other bases besides adenine have been isolated. The coenzyme from the horse heart was shown to be destroyed by alkali at the same rate as the coenzyme from horse erythrocytes. Hence the two coenzymes are probably identical. The addition of the erythrocyte coenzyme to cozymase-free Lebedew yeast juice does not restore the ability of the juice to ferment sugar.

#### CARBOHYDRASES

*Amylase*.—Malt extract may be purified threefold by ultrafiltration according to Snell (90). The membranes adsorb some of the enzyme.

Sherman, Caldwell & Doebbeling (91) describe a method for the purification of malt amylase. The purified product, which is more active than any previously reported, contains 16 per cent nitrogen and behaves like a typical protein. Denaturation of the protein coincides with inactivation of the enzyme. The amylase has the properties of a  $\beta$  amylase.

"Sistoamylases" have been found by Chrzaszcz & Janicki (92) to be present in germinated and ungerminated cereals as well as in animals. The sistoamylases inhibit animal and plant amylases by adsorbing them. Peptone reactivates and is said to act as an "elutoamylase."

Hollander (93) finds that rat-liver-amylase preparations differ greatly in their ability to digest starch to a blue-violet end-point and in their ability to form maltose. In a few cases the end-point was reached without any formation of reducing material. Incubation of aqueous liver suspension increases its ability to form maltose. The hypothesis is that there are present two amylases and an unstable inhibitory substance. The inhibitory substance can be precipitated by acetic acid.

Active papain increases the amylolytic power of barley and other grains with the exception of millet which becomes less active. The effect upon barley is either due to a production of more amylase or to a setting-free of amylase bound to protein (94).

*Emulsin*.—Albers & Hamann (95) find that the synthesis of mandelic acid nitrile by emulsin is of the second order and that the velocity



shows a linear relationship to the amount of enzyme employed. This is independent of the purity of the enzyme. An oxynitrile unit is defined.

Albers & Hamann (96) find that the impurities in oxynitrilese have no effect upon the activity-pH curve. This curve has maxima at pH 5.4, 5.85, and 6.5.

Ionescu & Kizyk (97) have cleared up two puzzling points in connection with synthesis and hydrolysis by  $\beta$  glucosidase. First, they have confirmed previous experiments which found the equilibrium constant to vary with alcohol concentration, where:

$$\frac{[\text{methanol}][\text{total glucose}]}{[\text{glucoside}][\text{water}]} = K = 0.237 \text{ for 10 per cent alcohol,} \\ \text{changing to 0.283 for 90 per cent.}$$

When, however, the concentration of  $\beta$  glucose instead of total glucose is used, a true equilibrium constant  $K^1 = 0.149$  is obtained which is independent of the alcohol concentrations.

Secondly, they have confirmed previous experiments which showed that the velocity of synthesis and hydrolysis were equal in 30.2 per cent alcohol when the concentration of glucose and glucoside were the same.

Since  $V_s = K_s [\text{alcohol}] [\beta \text{ glucose}]$  and  $V_H = K_H [\text{glucoside}] [\text{water}]$ , at equilibrium  $V_s = V_H$  and  $K^1 = \frac{K_H}{K_s} = 0.149$ . The fact

that the two velocities were equal but that the equilibrium constant was not equal to 1 had puzzled previous workers and led them to suppose that the law of mass action did not apply to this reaction.

*Saccharase*.—Lutz & Nelson (98) find that saccharase prepared by the toluene autolysis of yeast at pH 7 is totally insoluble in saturated ammonium sulfate, while if prepared at pH 5 it is largely soluble after preliminary purification. The saccharase soluble in saturated ammonium sulfate does not appear to contain protein. It is as active as any saccharase yet described.

Nelson & Saul (99) found that egg albumin, edestin, globin, and serum accelerate impure saccharase at pH 3, but not at pH 4.5. Denatured proteins did not affect the activity of highly purified saccharase at pH 3.

By toluene autolysis of fresh *Schizo-saccharomyces octosporus* Hofmann (100) obtained a solution which split maltose, but which did not have the slightest action upon sucrose. This is believed to disprove the theory of Weidenhagen of the identity of maltase with gluco-saccharase.

## AMINOCYCLASES

*Urease*.—Howell & Sumner (101) find that the activity of crystalline jack bean urease depends upon the type of buffer employed as well as on other factors. For 2.5 per cent urea and 0.125 *M* buffer the pH optimum in acetate is at 6.4, in citrate 6.5, and in phosphate 6.9. For 0.1 per cent urea it is 6.7 in acetate, 6.7 in citrate, and 7.6 in phosphate.

Fishgold (102) finds that while quinone inhibits urease, after reducing quinone with active hydrogen there is no inhibition. The inhibition by quinone is thought to be due to a chemical reaction with the urease rather than to a general effect upon the oxidation-reduction potential, since ferricyanide with a potential of more than +500 m.v. does not inactivate urease, whereas quinone with only +200 m.v. does.

*Arginase*.—It had been believed previously that the -SH group (103) was necessary for the activation of arginase but Purr & Weil (104) find that the -SH group is not necessary, since ascorbic acid-Fe-, methyl glyoxal-Fe-, and alloxan-Fe- also activate arginase. They believe that a certain oxidation-reduction potential is necessary. This potential varies with pH.

Arginic acid ( $\alpha$ -hydroxy- $\delta$ -guanido-valeric acid) is readily hydrolysed by arginase, as found by Calvery & Block (105), hence the amino group of arginine is not essential for arginase action. The finding of Edlbacher & Bonem that the methyl ester of arginine is not split by arginase has been confirmed, hence arginase probably requires a free carboxyl group in arginine.

Karrer & Zehender (106) find that the actions upon arginase activity of cysteine, ascorbic acid, dehydroascorbic acid, lactoflavin, and metallic ions are due to very complicated processes. Ferrous ions activate; ferric ions activate less strongly; calcium ions are ineffective in low concentrations, but in high concentrations inhibit; cupric ions act like calcium ions. Cysteine alone at pH 9.3 inhibits. This inhibiting action is removed by small concentrations of ferrous or ferric ions. In large concentrations ferrous and ferric ions activate strongly. Large amounts of calcium or cupric ions inhibit arginase-ascorbic acid strongly, or completely. The action of dehydroascorbic acid is similar to that of ascorbic acid, only here the activation by ferrous and ferric ions is smaller. Lactoflavin inhibits arginase.

Leuthardt & Koller (107) point out that all activators of arginine are reducing agents, that arginase with cysteine is never more active than arginase in the absence of oxygen, that arginase from fresh pig

liver is not activated by cysteine, and that those samples of arginase that are capable of activation by cysteine are the ones which are sensitive to oxygen. They conclude that the activation of arginase is a reduction process.

Weil & Russell (108) have described a micro-method for arginase. The urea formed by arginase is decomposed by urease and the liberated carbon dioxide is determined manometrically.

*Desamidase*.—Neuenschwander-Lemmer & von Leövey (109) find natural alanine to be deaminized with difficulty by rat kidney desamidase, while *d*(-)-alanine is readily deaminized. Whether the difference is due to difference of affinity of the enzyme to the two substrates or to a difference of velocity of decomposition of the substrate-enzyme could not be decided.

#### ESTER-SPLITTING ENZYMES

*Lipase*.—The hydrolysing actions of rat, horse, sheep, ox, and human sera upon 10 different esters have been followed (110). Definite differences exist in different species. Horse serum was the most active.

Sobotka & Glick (111) have studied the pH-activity curves of human and hog-liver esterase and hog pancreatic lipase, using various esters. With all esters used except triacetin the curves showed a dip at about pH 7.5 when the buffer mixtures contained diammonium phosphate. No dip was observed when other buffers or purified enzyme preparations were employed.

Bamann & Laeverenz (112) state that they have obtained from the pancreas a "crystalline lipase protein," but do not present evidence which would enable one to consider how this is related to the lipase.

According to Kuntara (113) the lipase of the intestinal juice of *Helix pomatia* has its pH optimum at 6.3 to 6.7. The temperature optimum is at 35° C. Albumin inhibits; calcium oleate and albumin inhibit almost completely. The lipase of the mesenteric gland has a pH optimum at 9.0 and at this pH the enzyme is not inhibited by calcium oleate and albumin.

Glick (114) describes a micro-method for lipase activity. The substrate is methyl butyrate. The buffer is glycoll-sodium hydroxide at pH 8.7. The digest is titrated to pH 6.5 with 0.05 *N* hydrochloric acid in the presence of phenol, using bromthymolblue.

*Esterase*.—The asymmetric hydrolysis of ethyl-*d*-*l*-mandelate by liver esterase is found by Bamann & Laeverenz (115) to be hardly

affected by calcium oleate and albumin. Sodium oleate does influence the reaction, while the addition of calcium chloride inhibits this effect.

Ammon & Bartscht (116) describe a new type of dilatometer for the study of ester formation and hydrolysis. When butyl butyrate is synthesized by pancreas powder a linear relationship exists between increase in volume and decrease in butyric acid concentration. On the contrary, during hydrolysis, it is not possible to follow the reaction quantitatively, although a decrease in volume is noted.

*Phosphatase.*—Hansen (117) finds, after death from *osteogenesis imperfecta*, almost complete absence of phosphatase in the periosteum and sub-periosteal structures and low values for the duodenum.

Bodansky & Jaffe (118) have observed that after ligation of the common bile duct in the dog, serum phosphatase may increase as much as 80 fold.

Palmer & Nelson (119) find little choice between the Bodansky and the Jenner-Kay methods for the estimation of phosphate activity.

Common (120) finds serum phosphatase to be increased in fowls during the periods of laying and moulting, although the values are variable.

Iwatsura & Minami (121) find that in acute leucaemia the serum phosphatase has the same activity as that of normal blood. In chronic myeloid leucaemia it is increased. The phosphatase of liver, spleen, and kidney is also increased in myeloid leucaemia. Leucocytes which do not give the oxidase reaction are found to contain no phosphatase.

Bodansky (122) finds that alimentary hyperglycemia is accompanied by an increase of serum phosphatase.

Davies (123) finds spleen and liver phosphatase to be different from bone phosphatase and from erythrocyte phosphatase; it shows two pH optima. It is suggested that spleen and liver contain an enzyme identical with bone phosphatase and another enzyme different from either bone or erythrocyte phosphatase.

Macfarlane, Patterson & Robison (124) describe the estimation of bone phosphatase, using tissue fragments. The bladder contained phosphatase in all species examined; the trachea contained phosphatase in most species; the aorta contained phosphatase only in rats.

*Phosphocreatinase.*—An enzyme has been shown by Lohmann (125) to bring about the splitting of phosphocreatine. Here magnesium ions are not necessary, but adenosine triphosphate is required. The reactions are:



Adenylic acid + 2 phosphocreatine = adenosine triphosphate + 2 creatine.

*Phospholecithinase.*—The enzyme previously found in the kidneys and intestinal mucosa, which splits phosphoric acid from lecithin, is now shown by King (126) to attack hydrolecithin as fast as lecithin and to attack cephalin and phosphadic acid more slowly. Synthetic lecithin and distearyl phosphate are attacked at a still lower rate, but brominated lecithin is split more rapidly than lecithin.

#### OTHER ENZYMES

*Lysozyme.*—Meyer, Thompson, Palmer & Khorazo (127) describe lysozyme, previously studied by Fleming (128). It occurs in tears, nasal, bronchial, and gastro-intestinal mucus, egg-white, and semen. It splits a reducing sugar from certain mucoids.

Mitsunori Wada (129) finds an enzyme in blood, milk, pancreas, and liver which splits urea from carbamino acids, hydantoins, and proteins.

*Brain enzymes.*—Edlbacher *et al.* (130) find that brain tissue splits phosphoric acid from nucleic acid and from the magnesium salt of hexose diphosphoric acid only weakly. It does not attack glycerophosphate at all. It has a weak catheptic action and no tryptic action. It has a weak lipase action. No difference was observed between white matter and a mixture of white and gray matter.

*Distribution of enzymes in the pig stomach.*—Linderstrøm-Lang and co-workers (131) have determined the pepsin, peptidase, esterase, and acid in pig-stomach mucosa at different depths.

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## THE CHEMISTRY OF THE CARBOHYDRATES AND THE GLYCOSIDES\*

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### INTRODUCTION

The reviewers who were responsible in 1933 for summarising the advances in sugar chemistry prefaced their account by the well-justified plea that their task had not been easy. In the intervening two years, activity in this field of research has continued unabated and the most rigorous selection of meritorious publications has produced a mass of material of unwieldy dimensions. An attempt has therefore been made to sketch a picture showing the general lines on which carbohydrate chemistry is now being developed rather than to synthesise individual researches. The idea has been kept strictly in mind that problems of absorbing interest to the orthodox organic chemist may be remote from those of immediate importance to the biochemist and in consequence some investigations which would find a place in a review with a different objective have been excluded. No attempt has been made to include border-line biochemical investigations of a type not readily interpreted in terms of molecular structure; this treatment of the subject has its limitations but under the circumstances it is inevitable.

Taking a broad view, it is evident that constitutional studies of the natural carbohydrates are entering on a new phase. The oxidic rings of the important sugars have been characterised, the linkages which unite simple saccharides to form the higher saccharides have been identified, and, although the structure of typical polysaccharides may be regarded as still unsettled, progress is being made and divergences of view are concerned with detail rather than with gross structure. If, as seems likely, conventional methods of attack must now be supplemented by enquiries on physical and biological lines it seems improbable that our present ideas of carbohydrate structure will be profoundly altered for some time to come. To have a halting stage in purely constitutional studies will in no sense react to the disadvantage of sugar chemistry and will afford an opportunity for interpreting biological changes in terms of structure and for testing

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accepted structures through their capacity to afford a rational explanation of biological reactions. The smooth syntheses by which the complex saccharides are built up in Nature from simpler units; the equally astonishing natural processes whereby, with the minimum of energy change, sugars are oxidised, reduced, or degraded; the facility with which through configuration changes one sugar is transformed into another—these are all problems of immense scope and of special importance to the biochemist.

Fortunately signs are not lacking that the attention of chemists is being attracted to these problems and to the mechanism of the fundamental reactions of which sugars are capable. We may therefore look forward to a period when dramatic discoveries will be few in number, when more attention will be paid to the specific properties of the individual hydroxyl groups in sugars, and when the biochemical distinction between one sugar and another will be studied in terms of the configuration of the asymmetric systems which form the carbohydrate chain.

These are generalisations, the significance of which it is hoped will be apparent in the references which follow under separate headings.

#### SIMPLE CARBOHYDRATES

The practical worker in this field must welcome the many improvements in methods of preparation which have been recorded recently and also the use of novel exploratory reagents and new analytical processes. Personal experience is required to assess the value of these innovations, but a few outstanding examples may be quoted as of general interest. The preparation of *d*-arabinose from calcium gluconate by oxidation with hydrogen peroxide and ferric acetate is a striking case of the control now exercised over a reaction which at one time was notoriously fickle (1). *l*-Ribose, *d*-allose, and *d*-xylose are other sugars to which access is now easier (2, 3, 4), while the preparation of mannose has been much simplified (5) as has also the preparation of *d*-galacturonic acid (6). A synthetical variation of the latter preparation is provided by oxidising diacetonegalactose with potassium permanganate followed by removal of the *iso*-propylidene residues (7). In the preparation of lactones related to the sugars, working processes have now reached a high standard of efficiency (8, 9, 10).

Efforts to find a reagent sharply discriminative between the primary and secondary alcohol groups in the simple sugars have not been

so successful as at one time seemed probable. For example, triphenylmethylchloride reacts normally with  $\alpha$ -methylglucoside to give a monotriyl-derivative in which the primary group is substituted but the reaction can be extended also to secondary groups (11, 12). The reagent no doubt attacks primary groups preferentially but the reaction can no longer be regarded as diagnostic; the example quoted does not stand alone and the completely discriminating reagent is still to be found.

Structural studies of the simple sugars have proceeded on normal lines and only a few papers call for special comment. Levene and his co-workers have applied what may be termed the "absolute method" of confirming the position of the oxygen-ring in  $\gamma$ -glucosides by the observation that whereas 4-methylglucoheptose gives a normal methylglucoside only, 2-methylglucose and 3-methylglucose share with the parent sugar the capacity to form both normal and  $\gamma$ -glucosides (13). It is now recognised that one of the first points to be settled in the constitutional study of any reducing sugar is the position of the oxygen-ring; in view of the insignificant part played by  $\gamma$ -aldoses in Nature, it is interesting to note that the ribose residues in yeast nuclei are now shown to possess the furanose structure (14, 15). The temptation to regard all reducing sugars as 1,4- or 1,5-oxides must, however, be avoided; other possibilities cannot be ignored. For example, two new pentacetates of *d*-galactose have been described and to each of these compounds a 1,6-ring can reasonably be assigned (16, 17), a conclusion which is not surprising in view of the formation of anhydrides in which the same two positions are coupled through oxygen.

Recent studies in mutarotation have given results which serve still further to emphasise the need for caution; no longer can these optical changes be interpreted as due exclusively to the establishment of equilibrium between  $\alpha$  and  $\beta$  forms. In the case of arabinose it is now shown that three modifications exist in equilibrium (18) and the same holds true for ribose (19). This result does not necessarily prove the simultaneous existence in solution of sugars possessing different oxygen-ring systems, but it is very probable that such is the case.

Mutarotation in aqueous solution is already sufficiently complicated, yet now we must face the additional factor introduced by using "heavy" water as the solvent (20, 21). In  $D_2O$ , although the optical end point attained by glucose is scarcely if at all affected, the speed of the reaction is sharply depressed; it is a sobering thought that some

day the chemistry of the sugars may have to take account of the energy content of each hydrogen atom in each hydroxyl group.

The chemist has occasionally to remind himself that some reactions of reducing sugars are still best explained in terms of the aldehydic and ketonic structures formerly in use. Aldoses and ketoses are not always ring compounds, and importance must therefore be attached to the work of Wolfrom and his colleagues on the preparation of open-chain sugars. Galactose and fucose have been added to the list of sugars which can give true aldehydic derivatives (22, 23) and a more important case is presented by " $\alpha$ -pentacetylfructose." This compound is apparently devoid of any oxygen-ring and is regarded as the pentacetate of keto-fructose (24, 25). It is possible that the particular form assumed by a reducing sugar in solution is determined in large measure by the pH value. Thus, one of the spectral bands shown by alkaline glucose solutions is attributable to the presence of the aldehydic form but this characteristic band vanishes as the alkalinity is diminished (26, 27). As additional examples showing how the reactivity of a sugar is dependent on the pH it may be noted that the stability of fructose is maximum when the value is 3.3 (28) and that change in pH definitely alters the proportions of the isomeric forms of reducing hexoses which have attained equilibrium (29). This in turn must affect the mechanism of many sugar reactions of biological importance, including oxidation (30, 31). Further developments in this type of research will be awaited with interest.

Enzymes continue to play an important part as diagnostic reagents in carbohydrate research. A good example of their application is found in the action of invertase on what can only be described as "the  $\gamma$ -methylfructoside mixture." As is well known the condensation of fructose and methyl alcohol is complex and gives rise to syrupy derivatives of the  $\gamma$ -type which are non-separable by ordinary agencies. A distinct advance has been made by acting on such mixtures with invertase which is capable of fermenting the components possessing the  $\beta$ -configuration. In this way it has been shown (32) that the non-fermentable residue contains three fructosides and the research has resulted in the isolation of a new crystalline methylfructoside presumably of the  $\gamma$ -type. Much importance must be attached to this investigation which may well have far-reaching results. Other researches in which specific enzymes are employed and where constitutional issues are involved include the decomposition of inulin by inulinase (33) and by  $\beta$ -fructosidase respectively (34), but the evi-

dence forthcoming does not appear to determine finally whether fructose is the only hexose which builds up the polysaccharide molecule. The general retarding effect on the reactions of glucose by the use of deuterium water as a solvent is further emphasised by the observation that the speed of alcoholic fermentation is thereby depressed to one-ninth of the normal, but in this case the result appears to be due not so much to any effect on the sugar as to the partially destructive action of the solvent on the enzyme (35).

*Anhydrides.*—The tendency to form anhydrides has long been recognised as a common property of sugars and many such compounds have been described in the past with a confidence scarcely justified. In the great majority of cases these products were complex uncrystallisable mixtures and evidence did not exist as to which hydroxyl groups had taken part in their formation. Now that it is experimentally possible to protect selected hydroxyl groups of a sugar chain and to carry out reactions on the remaining free hydroxyl groups the whole problem of anhydro-formation has assumed a new aspect and has acquired exactitude.

In two respects investigations on sugar anhydrides are important to the biochemist. Such compounds occur naturally and in addition it will no doubt be found that one of the most fruitful sources of Walden inversions in the sugar group resides in the successive formation and opening of anhydro-rings.

Two new examples of natural anhydrides related to the sugars may now be added to the list, as styracitol and polygaritol have been proved to be respectively 1,5-anhydrosorbitol and 1,5-anhydromannitol (36). New artificial anhydrides of mannitol have also been described (37) and the polyhydric alcohols evidently offer abundant scope for this type of research.

Among the common reducing sugars, fructose is notoriously prone to undergo molecular dehydration even under mild conditions. It is now recorded that under the influence of hydrogen chloride in the cold the ketose is converted into a new form of difructose anhydride (38) in which the hydroxyl groups 3, 4, and 5 remain unsubstituted, a somewhat unexpected result. In this connection it will be recalled that the acetolysis of inulin gives a small amount of the acetate of a different difructose anhydride and the question at once arises if this is a genuine scission product or is derived in a secondary reaction from the fructose liberated on hydrolysis. It is not yet certain which interpretation is correct although it is important to note that the



hydrolysis of trimethylulin similarly gives a small proportion of a methylated difructose anhydride which apparently does not originate in dehydration of trimethylfructose.

Very naturally, in view of its relationship to the polysaccharides, 2,3,6-trimethylglucose has been used as a test substance upon which to study dehydration phenomena, but the results so far obtained in this enquiry are contradictory for reasons which are still obscure (39, 40, 41).

A new form of anhydro-galactose has been described in which ring-formation is claimed to take place between positions 3 and 6 although a rigid proof of the structure is still lacking (42). Greater interest is attached to the ingenious method whereby acetobromodisaccharides can be converted into anhydro-derivatives and the following characteristic products have now been obtained in this way:

- a) 4- $\alpha$ -glucosido-glucosan from maltose
- b) 4- $\beta$ -glucosido-glucosan from cellobiose
- c) 4-galactosido-glucosan from lactose (43, 44).

*Transformation of isomeric sugars.*—It will be generally admitted that the mechanism involved in the smooth transformation of one simple sugar into another is a matter of the utmost importance alike to the chemist and to the physiologist. Recent examples of the conversion of aldoses into the corresponding ketoses (45, 46) by the agency of pyridine encourage speculation on the use of this reagent in the general exploration of the problem and the possible effect of pH on the change, but it would be necessary to modify the drastic conditions of such experiments before the results can have biological significance. Another avenue of thought is opened with the instance of the conversion of galactose into talose (47) by addition on the double bond in galactal, but the general application of such a principle is circumscribed, although another successful case is quoted in which maltal is converted into 4- $\alpha$ -glucosidomannose (48).

At the moment it would appear that the most hopeful way of approaching the problem lies in a consideration of the means by which Walden inversions may be induced within the simple sugar molecule. In this connection it has recently been shown that the alkaline hydrolysis of 2,3-di-*p*-toluenesulphonyl derivatives of glucose leads to the production of 2,3-anhydro-derivatives along with derivatives of an isomeric hexose (49, 50). In all the cases which have been studied, the formation of the isomeric hexose is accompanied by anhydro-formation and this fact emphasises the suggestion already made that

inversion follows as a consequence of the opening of the anhydro-ring.

It is true that such conversions are accomplished under conditions remote from those which prevail in the plant or animal body, but the mechanism involved may nevertheless be similar. Knowledge in this particular realm of carbohydrate chemistry can scarcely be regarded at this stage as more than qualitative, but exploration on such lines may lead to results of fundamental importance.

#### PARTIALLY SUBSTITUTED CARBOHYDRATES

Greater difficulty has been experienced in reviewing this class of derivative than in any other section of the carbohydrate report. Biochemists have for long recognised that in interpreting the natural reactions of sugars it is necessary to think in terms not of gross molecules but of individual hydroxyl groups, and a development which is gradually adding to our knowledge of how each of these groups reacts must be welcome.

Experimental researches designed to this end depend on a few principles of general application. For example, certain pairs of hydroxyl groups can be occupied by condensation with acetone, or benzaldehyde, or with boric acid, thereby leaving other hydroxyl groups free for attack with different reagents. This does not exhaust the methods of access to partially substituted sugars, and opportunities for research of this kind are almost limitless. Many recent publications reveal the ingenuity and labour devoted to the preparation of sugar derivatives of the most diverse nature in which the composition and position of the substituting groups have been extensively varied. But the time is not yet ripe to draw generalisations of any permanent value and in some cases it is even impossible to recognise the ultimate objectives of the work. Meanwhile, the situation resembles that of an incomplete jig-saw puzzle in which the key pieces have not yet been inserted and in which the picture remains unrevealed. Reference is accordingly restricted to publications in which definite conclusions have been reached; good examples are furnished by elaborate studies of the 4-position in glucose and of the 5-position in xylose, for the details of which the original papers should be consulted (51, 52, 53).

Brigl and his co-workers have been responsible for a series of papers dealing chiefly with various benzal derivatives of hexoses and with the benzoates derived from them (54, 55), while other investigators have shown a tendency to concentrate on the acetone sugars

as the source of the derivatives they describe (56, 57, 58). In all work of this description it is clearly necessary to check the constitution of the initial compounds carefully and to be on continual guard against the possibility of migration of the substituting groups. In this connection it may be mentioned that the constitution assigned by Levene & Meyer to 5,6-benzylideneglucose has been subjected to a challenge (59) which proved to be altogether unnecessary (60). Also, the observation that benzaldehyde condenses in two different ways with  $\alpha$ -methylmannoside should not be overlooked in future work on this hexose (61).

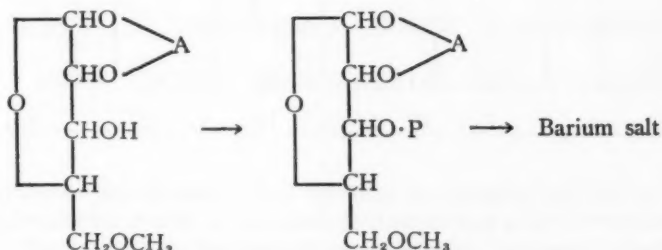
*Phosphates.*—The study of carbohydrate phosphates continues to be developed with a precision worthy of the importance of these compounds, and the examination of natural phosphates is being supplemented by exact syntheses. Before turning to the pentose phosphates, upon which at present interest is focussed, it may be mentioned that it is readily possible to obtain the 3-acetyl-6-trityl derivative of monoacetoneglucose and that the vacant hydroxyl group in position 5 can be phosphorylated. Ultimately, through the medium of the barium salt, glucose-5-phosphate can be isolated and evidence is forthcoming that this particular derivative is not an intermediate product in the alcoholic fermentation of glucose (62). A definite mannose phosphate has also been described (63), and yet another reference compound in this field of research is now available through the preparation of the monophosphate of dihydroxyacetone (64).

Great interest is to be attached to the pentose phosphates which are being investigated by Levene and his collaborators. As a result of a series of interdigitated researches these workers have contributed a very neat proof that the ribose phosphoric acid from yeast adenylic acid (which is identical with that obtained from guanylic acid) carries the phosphoric acid residue in position 3 and, in consequence, it is now possible to describe adenylic acid as adenine-*d*-ribofuranoside-3-phosphoric acid (65). In an extension of the research, an isomeric ribose phosphate containing the acid residue in the 5-position has been synthesised (66) from methylriboside by the scheme represented below: Monoacetone methylriboside  $\rightarrow$  monoacetone methylriboside monophosphate  $\rightarrow$  (via the barium salt) ribose-5-phosphoric acid.

The structural interpretation of the above reactions demanded a separate investigation of the condensation of *d*-ribose with acetone, a reaction which yields only a single derivative in which the *iso*-propylidene residue occupies positions 2 and 3 (67). It may be noted

that the procedure of subjecting these pentose phosphates (or their salts) to reduction as a means of throwing additional light on their structure is now firmly established and is giving interesting results (68, 69).

Meanwhile, attention has rightly been given to the possibility that the introduction, and more especially the elimination, of the phosphoric acid residue may be accompanied by change of configuration in the sugar chain, thereby vitiating the constitutional evidence obtained by hydrolysis or furnished by synthesis. A test case has been provided, the initial compound being the monoacetone derivative of 5-methylxylose which was subjected to the cycle of reactions shown below where  $A$  = the acetone residue and  $P$  = the phosphoryl residue:

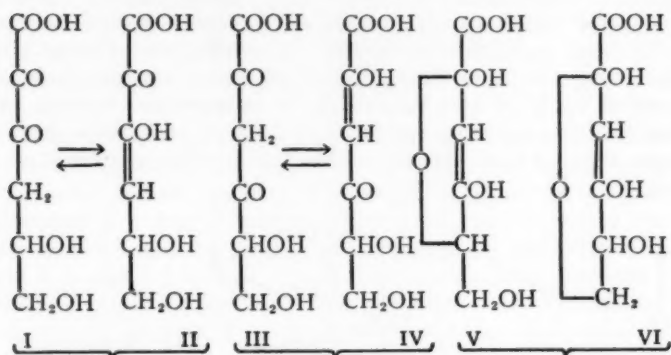


Consecutive phosphorylation and dephosphorylation were completed without change in configuration, thereby affording evidence that the *d*-ribose obtained from nucleic acids is a genuine constituent of these complexes and is not a secondary product formed through a Walden inversion operating on position 3 of the pentose chain (70).

*Methylated sugars.*—The process of methylation continues to play a conspicuous part in researches designed to elucidate the structure of carbohydrates, and the corresponding literature is in process of being collected (71). With the exception of improved working methods (72, 73) and the series of reactions in which the important reference sugars 2,3,4- and 2,3,6-trimethylglucose have been obtained simultaneously (74), there are no other outstanding results suitable for report under this heading.

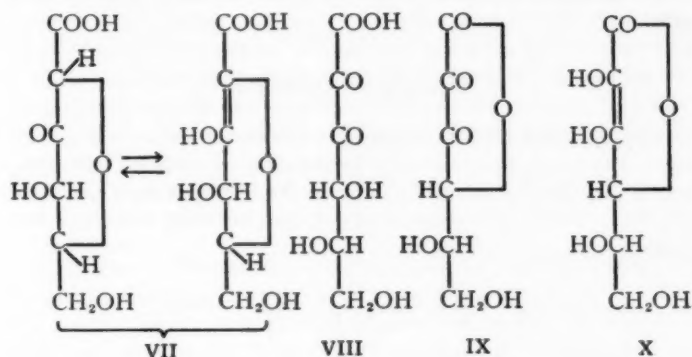
*Ascorbic acid.*—After many fluctuations of opinion the difficult problem of the constitution of ascorbic acid has been brought to a successful issue and the conclusions arrived at have been established

by synthesis. The early work of Hirst and his collaborators (75) pointed to ascorbic acid possessing the optional formulae I and II shown below, while researches conducted simultaneously under Karrer (76) favoured formulae III and IV which were afterwards modified to V and VI by the introduction of oxygen-rings.



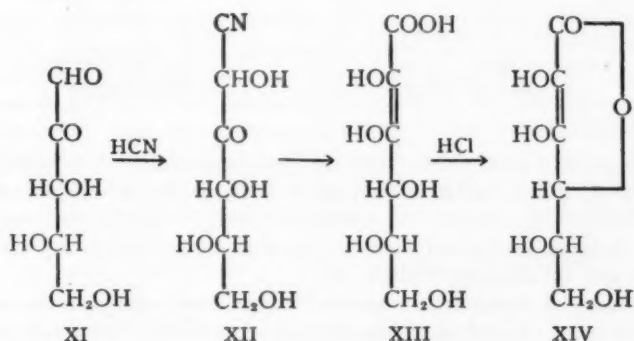
A striking advance was made by Karrer through the discovery that ascorbic acid reacted with diazomethane to give a dimethyl derivative. Practically simultaneously the same methylation was carried out by Micheel & Kraft (77), whose further work on the action of ozone on a fully substituted ascorbic acid contributed in large measure to the speedy elucidation of the structural problem. The accumulated evidence led them to adopt a cyclopentane structure VII. A consistent scheme could thereby be formulated for the reactions of ascorbic acid and it was accordingly a bold step to dispose of the idea that the acidic properties of the compound were due not to the presence of a carboxyl group but to an activated hydroxyl group. This view was adopted by Hirst who also observed that dimethyl ascorbic acid reacted with sodium hydroxide without the elimination of methyl alcohol. This observation had eluded Karrer & Micheel. Moreover, the trihydroxybutyric acid obtained from ascorbic acid was shown to be *l*-threonic acid which at once related the stereochemical configuration to *l*-gulose (78). Further, the reversible oxidation product obtained from ascorbic acid by the action of aqueous iodine yielded the same threonic acid on oxidation, thus confirming the view that it could react in the form VIII although at the time of its formation

it appeared to exist as the lactone IX. This led to the idea that X was the structural basis upon which the properties of ascorbic acid were to be interpreted and that its formula was to be derived by the abstraction of an atom of oxygen from VIII, or by the addition of two atoms of hydrogen to the lactone IX. Various tautomeric modi-



fications are possible, but in a final investigation on the methylated derivatives of ascorbic acid proof was afforded for the formulation of di- and tetra-methyl ascorbic acids and also for the product of ozonisation (79), so that the most probable structure for ascorbic acid is represented by X (80).

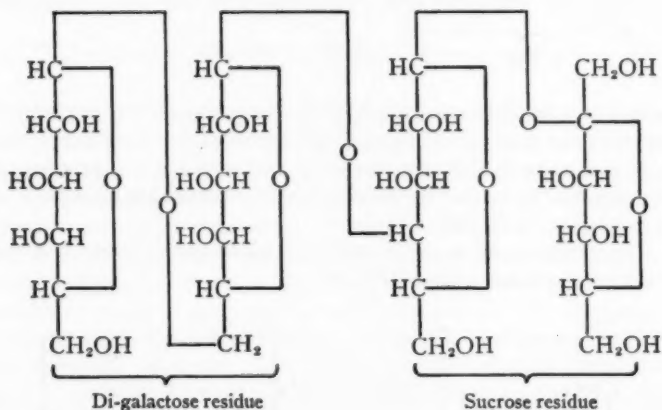
The synthesis of ascorbic acid was based on xylosone and the successive steps are indicated below:



Although Reichstein and his collaborators (81) were the first to isolate a synthetic derivative of *d*-ascorbic acid, the interpretation now given is due to Haworth and his collaborators (82), who succeeded in isolating crystalline specimens of *d*- and *l*-ascorbic acid. These methods have been extended to the preparation of analogues of the compound, and the subject, generally, is being closely investigated.

### HIGHER SACCHARIDES

Constitutional studies designed to determine the linkage of one sugar with another are naturally becoming fewer, but an interesting case is provided by the methylation of the tetrasaccharide stachyose (83, 84). The results point clearly to the following structural formula:



Whereas the glucose residue conforms to the usual model, the coupling of one galactose residue is unusual, as is shown by the isolation of 2,3,4-trimethylgalactose as a scission product. It may be mentioned that the same methylated sugar has been prepared from gum arabic (85) and has been synthesised (86).

Otherwise, research on *n*-saccharides has been largely confined to specific reactions and to various attempts at synthesis; but it may be

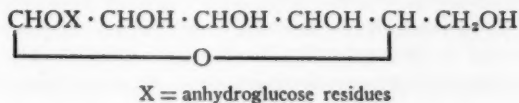


pointed out that the expression "synthesis" is loosely applied and is not confined, as it ought to be, to the formation of a compound from an origin of known constitution through a series of reactions each stage of which can be interpreted in terms of structure. The synthesis of cellobiose by Freudenberg & Nagai (87, 88) conforms to this definition as do also the efforts now being made to introduce amino acid residues into selected positions in the glucose chain (89, 90). The claims that the synthesis of sucrose has been effected by condensing together the appropriate tetracetates of glucose and fructose are not further supported by recent work on this subject and it will be generally agreed that some other route to this "unattained goal of sugar chemistry" must be found.

### POLYSACCHARIDES

*Cellulose.*—Although the increasing use of cellulose derivatives in industry is clearly reflected in the recent literature the purely scientific study of the polysaccharide retains a prominent position. It is well that this is so, for despite steady progress the elucidation of the cellulose structure in terms of molecular constitution is far from complete. Much recent work on this subject depends on the methylation of cellulose by methyl sulphate and sodium hydroxide. When it is recalled that this process was first applied twenty-five years ago by Denham who succeeded in isolating and identifying 2,3,6-trimethylglucose from a methylated cellulose, subsequent progress by other workers using the same agency must seem disappointingly small.

One additional result has, however, emerged through the experimental modification of starting with acetylated cellulose in place of the unsubstituted polysaccharide. Under these conditions the hydrolytic product has been shown to contain detectable quantities of 2,3,4,6-tetramethylglucose. Assuming that no molecular rupture takes place in the successive processes of acetylation and methylation, the presence of this sugar is opposed to the idea that cellulose is exclusively composed of anhydroglucose residues. So elementary a structure for cellulose as this was at no time upheld as it ignores several factors, and account must be taken of the presence of the fragment



Much more remains to be done. It must not be overlooked that cellulose is notoriously prone to undergo degradation with the development of reducing properties when treated even in the cold with powerful reagents. In consequence the amount of tetramethylglucose which may be obtained from cellulose varies greatly according to the experimental conditions employed (91); this renders uncertain calculations of the molecular weight which are based on the yield of this sugar. Efforts have been made (92) to minimise the risk of such adventitious hydrolysis interfering with the result, and the conclusion drawn is that from 100 to 200 glucose residues linked through the 1,4-positions represent the limits of the cellulose molecule. The actual length of the chain is largely a matter of academic interest.

That the isolation of tetramethyl glucose need not of necessity give an index of the length of the chain is shown by a careful study of a standard  $\alpha$ -cellulose from wood (93). The hydrolysis of methylated wood cellulose reveals the presence of a fragment more resistant to hydrolysis than the major portion and the origin of the tetramethylglucose has been traced to the former constituent. Similar results were obtained from cotton cellulose and the present situation is therefore inconclusive. Much depends on how far the actual constitution of cellulose is altered by the reagents employed in these researches and long and patient investigation is still necessary. Meanwhile the orthodox methods of attack are being supplemented by X-ray examination of cellulose derivatives (94, 95) with the result, so frequently encountered, that the findings of the organic chemist are confirmed generally rather than extended in detail.

A series of cellodextrins has been subjected to the methylation process and thereby shown to have a chain length of approximately 20 glucose units (96, 97). The progressive breakdown of cellulose shows a tendency to be arrested at a stage where a definite cellotriose can be separated from the mixture of products. Although some doubt exists as to the real nature of this compound (98, 99) the study of these products is obviously important. In this connection the confusion which has long existed as to the nature of "procellose" and "celloisobiose" is well on the way to being dispelled; the former represents essentially the trisaccharide stage of degradation while the latter is in all probability an impure preparation of cellobiose (96).

*Starch.*—On the whole, the evidence so far collected is in favour of uniformity in the molecular structure of starches derived from different sources, but several papers have appeared which indicate

that there are fundamental differences. For example, the benzylation of potato starch gives an excess of the dibenzoate, the tribenzoate being produced only in limited yield. This result must be considered in conjunction with the further observation that whereas amylopectin gives a dibenzoate, amylose is quantitatively converted into the tribenzoate (100). On the other hand the chemical relationship between amylose and amylopectin must be exceedingly close as the maltose linking is preserved in each and the same hydroxyl groups are unsubstituted.

A further stage has also been reached in correlating the molecular structure of polysaccharides with the physical form of these compounds as laid down in Nature. The statement that both cellulose and starch are composed essentially of glucose residues united in such a way that hydroxyl groups in positions 2, 3, and 6 remain free gives no clue as to why cellulose assumes the fibrous form while starch does not. The suggestion that the  $\beta$ -type of coupling adjacent glucose residues favours the production of thread-like molecules while the  $\alpha$ -linking prevents this formation goes far to afford a rational explanation of the difficulty (101).

The application to starch of *p*-toluenesulphonic acid as a reagent has yielded interesting results. The reaction gives a tritosyl starch in which the substituting group in the 6-position can be replaced by iodine (102) but the substitution can be extended so as to give the corresponding diiodo-derivative (103). Of greater importance is the fact that tritosyl starch undergoes a species of bromo-acetolysis and in this way a substituted methylhexoside can ultimately be obtained by standard reactions (104).

*Glycogen*.—Although many workers are engaged in the study of this polysaccharide the objectives sought and the methods used are chiefly those of the physiologist. Strictly chemical investigations are few in number. The methylation of glycogen has been continued on standard lines (105) and it is confirmed that when the methylation is carried out directly on the polysaccharide the introduction of methyl groups is arrested when eight out of nine hydroxyl groups are substituted. This difficulty is not encountered, however, when the polysaccharide is acetylated as a preliminary to methylation (106) and under these circumstances as much as 9 per cent of tetramethylglucose accompanies the 2,3,6-trimethylglucose which is the principal sugar formed on hydrolysis.

It is possible that both methods of investigation are subject to

inherent error. In the one case prolonged and repeated treatment with methylating reagents of which concentrated alkali is a component may eliminate hydroxyl groups by anhydro-formation; in the other, the preliminary acetylation even when confined to the mildest conditions may initiate the degradation and hydrolytic changes which accompany acetolysis. In effect, the methylation is conducted not on the original polysaccharide but on a "regenerate."

*Inulin.*—The structure of inulin has proved a popular subject of research and so many baffling problems have emerged that the present position is decidedly obscure. The essential nature of inulin as an aggregate of anhydro- $\gamma$ -fructose residues was revealed many years ago, but references have appeared persistently in the literature that in addition to fructose small quantities of glucose are produced when inulin is hydrolysed. The simplest method of disposing of this awkward product is to assume the presence of a small amount of starch as a non-separable impurity in the inulin used; the alternative explanation is that the inulin molecule contains a glucose residue coupled with many fructose residues.

Light is now thrown on this fundamental problem, as in a thorough examination of the hydrolysis products obtained from purified trimethyl inulin a small proportion of trimethylglucose was isolated (107). It was also shown that this particular compound is formed in secondary reactions from the trimethylfructose which is the essential hydrolysis product. The change involves the ketose  $\rightleftharpoons$  aldose conversion under very simple conditions and has therefore a special interest, but the result must be studied in conjunction with the further observation (108, 109) that when purified inulin is hydrolysed by enzymes as much as 1.5 per cent of glucose can be detected in the product. These results suggest that no glucose fragment exists in the inulin molecule but that hydrolysis whether by acids or by enzymes occasions a partial transformation of fructose into glucose. If, on the other hand, inulin contains an aldose residue then the yield of glucose can be used as an index of the number of six-carbon chains present and points to the existence of no fewer than 70 such units and a molecular weight of about 11,000. An entirely different result is indicated by the fact that trimethyl inulin yields on hydrolysis about 3.7 per cent of tetramethyl- $\gamma$ -fructose from which a chain varying in length between 27 and 30 units can be postulated. Confronted with data indicating a molecular weight for inulin ranging from 11,000 to 5,000 (110) and with the problem still unsolved as to whether glucose

is or is not preformed in the inulin molecule the position is bewildering. Amidst so much that is confusing, one definite advance has been made through the proof that the trimethylfructose obtained from inulin (111) is homogeneous and consists exclusively of the 3,4,6-variety.

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## THE CHEMISTRY OF THE ACYCLIC CONSTITUENTS OF NATURAL FATS AND OILS\*

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### GLYCERIDES AND THEIR CONSTITUENTS

Steger & van Loon (1) studied the composition of quince-seed oil. Contrary to previous investigations no "hydroxyoleic acid,"  $C_{18}H_{34}O_8$ , could be found among the unsaturated acids. This fraction contained only oleic and 9,12-linoleic acids together with a small amount of 9,12,15-linolenic acid. The same authors reported an analysis of the seed oil of *Cassia occidentalis* (2). The component fatty acids of some Malayan seed fats have been examined by Hilditch & Stainsby (3). Solid fats from the following plants were studied: *Sterculia foetida*, *Palaquium oblongifolium*, *Nephelium*; tonka-bean oil was also investigated. Examinations have been reported on seed fats of six members of the *Palmae* family (4).

Roberts & Schuette (5) found linoleic acid (perhaps in two isomeric forms) as the predominant unsaturated fatty acid (70.4 per cent of the fat) in Wisconsin-grown tobacco-seed oil. According to Riebsomer & Nesty (6) pumpkin-seed oil contains palmitic, stearic, oleic, and 9,12-linoleic acids. One of the infrequent cases where a natural glyceride is composed of almost one acid only has been reported by Puntambekar & Krishna (7). Ninety-six per cent of the fat extracted from the kernels of the seeds of *Actinodaphne hookeri* consists of trilaurin, the remainder being chiefly triolein. This fat is recommended as a convenient source of lauric acid. Work has also been done on the composition of the oils of *Tectona grandis* seeds (8), of the kernels of peaches, brazil nuts, and apricots, and of the seeds of pears and horse-radish (9) and of various Japanese *Leguminosae* and *Theaceae* (10).

Flaschenträger & Wolffersdorff (11) found that the toxic principle of croton oil could be separated from the fatty oil by means of petroleum ether in which the former is insoluble. The component fatty acids of the oil are myristic, palmitic, stearic, arachidic, oleic, and linoleic acids. According to Heilbron, Moffet & Spring (12) the non-

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saponifiable matter of shea nut fat is composed of the hydrocarbon illipene,  $C_{30}H_{52}$  (m.p.  $63-64^{\circ}$ ), previously described by Kobayashi, of  $\beta$ -amyrin, of lupeol, and of an alcohol,  $C_{30}H_{50}O$  (m.p.  $109.5^{\circ}$ ;  $[\alpha]_D = -11.9^{\circ}$  in chloroform).

The composition of the glycerides of coffee-bean oil has been studied by Bengis & Anderson (13). The glycerides of fresh coffee and of roasted coffee (both fresh and aged) were compared. Roasting increased the amount of water-soluble volatile acids in the fat. The solid acids consist mainly of palmitic acid, with small amounts of stearic acid and a tetracosanoic acid. The unsaturated fatty acids (52 to 54 per cent of the total acids) are composed of equal parts of oleic and linoleic acids. The fat also contained an unsaturated, optically active hydroxy acid of high molecular weight; it seems to be a conjugated acid made up of higher unsaturated hydroxy acids, one of which belongs to the  $C_{18}$  series, since reduction with hydrogen iodide gives stearic acid together with another higher acid. There have been some more papers published on coffee-bean oil by other workers (14, 15).

The fatty oil of millet has been examined by Steger & van Loon (16). In the fat of barley rootlets Smeets & Ruppel (17) found glyceryl and ceryl esters of acetic, caproic, palmitic, stearic, oleic, linoleic, and linolenic acids, sitosterol, and a trace of ergosterol.

According to Vanin & Chernoyarova (18) the oil of *Coriandrum sativum* is composed mainly of the glycerides of 6-oleic acid. The polymerization of Chinese wood oil is the subject of a paper by Steger & van Loon (19). Thomas & Thomson (20) prepared  $\alpha$ -eleostearic acid from tung oil and  $\beta$ -eleostearic acid from wood-oil butter.

From the fatty oil of *Parinarium macrophyllum* (Néou oil) Steger & van Loon (21) isolated an isomer of eleostearic acid, probably identical with couepic acid formerly isolated from oiticica oil (22). The unsaponifiable matter of ketiau oil from the seeds of *Ganna motleyana* contains lupeol (23). Among studies of other unusual vegetable fats those on the oils from *Persea indica* (24), *Jatropha curcas* [Barbadoes nuts (25)], and "kaoliang" [*Andropogon sorghum* (26)] may be mentioned.

From the kidney fat of a cow, Zechmeister & Tuzson (27) obtained crystalline carotene (probably a mixture of the  $\alpha$ - and  $\beta$ -compounds). There were no xanthophylls present. Schuette, Garvin & Schwoegler (28) published a study of the composition and properties of the abdominal fat of the western range horse.

Klenk & Ditt (29) examined the liquid triglycerides of beef-heart muscle. The distribution of fatty acids was the following: 22 per cent  $C_{16}$ , 20 per cent  $C_{18}$ , and traces of  $C_{20}$  and  $C_{22}$  solid fatty acids; 12 per cent  $C_{16}$ , 45 per cent  $C_{18}$ , and 1 per cent  $C_{20}$  and  $C_{22}$  liquid fatty acids. The component fatty acids of rat body fats were studied by Banks, Hilditch & Jones (30).

Hilditch, Jones & Rhead (31) investigated the composition of the body fats of the hen. Sixty-five to sixty-eight per cent of the component fatty acids consisted of unsaturated acids. It is remarkable that 7 to 8 per cent of 9-palmitoleic acid could be isolated; this acid has been found previously in aquatic animals, diphtheria bacilli, lycoperidium spores, and rat body fats. Other articles on the egg fat and the depot fat of fowl have been published by Cruickshank (32) and by Brown & Sheldon (33).

Bosworth (34) examined the fatty acids isolated from a large amount of fat from human milk. The same author together with Sisson (35) studied the  $C_{20}$  fraction of the fatty acids from butter fat. Arachidic acid was not found, whereas arachidonic acid could be separated in the form of its octabromide. Dhingra (36) reported on the glycerides and component fatty acids of the milk fat of Indian camels.

The fat of the eel was studied by Wiehr (37). From the liver oil of "hiragashira" (*Scoliodon laticaudus*) Ueno & Iwai (38) isolated a new highly unsaturated fatty acid, "scoliodonic acid," to which the formula  $C_{24}H_{38}O_2$  is ascribed.

Fester & Bertuzzi (39) published a comprehensive study of the gland secretion of alligators. They isolated myristic and palmitic acids, a series of unsaturated acids (among which occurred, possibly, an isomer of oleic acid), cetyl alcohol, and small amounts of glycerol and cholesterol. There also was present a liquid substance with a strong aroma, "yacarol" (b.p.  $234^\circ$  at 757 mm.), containing 8 or 9 carbon atoms to which the provisional formula of 2,6-dimethylhepten-(2)-ole (7)  $[CH_3 \cdot C(CH_3)=CH \cdot CH_2 \cdot CH_2 \cdot CH(CH_3) \cdot CH_2OH]$  was assigned.

The liver oils of different species of sharks were examined by Tsujimoto (40) who succeeded in isolating a new unsaturated hydrocarbon, zamene,  $C_{18}H_{30}$  (41). The same author (42) obtained from the oil of "karasumi" (the ovary of gray mullet, *Mugil japonicus*), cholesterol, cetyl alcohol, and octadecenol [cf. Kafuku & Hata (43)]. Tsujimoto & Koyanagi (44) analyzed the fats from various Japanese

shell-fishes and reported the isolation of a sterol, conchasterol, from "hamaguri." Toyama & Tsuchiya (45) isolated gadoleic acid,  $C_{20}H_{38}O_2$ , from Japanese sardine oil, herring oil, and the liver oil of *Theragra chalcogramma*, and established the constitution of this acid, by ozonolysis, as that of a 9-eicosenoic acid (46). This is in good agreement with the results obtained by Takano (47).

Lovern (48) worked on the composition of the fats from various organs of the dolphin, the conger eel (*Conger vulgaris*) and the porpoise (*Phocoena communis*). It is worth mentioning that the depot fats of both the dolphin and the porpoise contain considerable amounts of isovaleric acid. The fatty acids from the larva fat of the beetle, *Pachymerus dactris*, were examined by Collin (49).

The composition of the fat of *Penicillium javanicum* has been studied by Ward & Jamieson (50). The fat was found to consist of the glycerides of palmitic, stearic, *n*-tetracosanoic, oleic, and  $\alpha$ - and  $\beta$ -linoleic acids. The same acids were also obtained by Strong & Peterson (51) from the fat of *Aspergillus sydowi*, the unsaponifiable matter of which contained ergosterol. The conditions of the formation of sterols and lipids by yeast were studied by Halden (52).

In continuation of their work on the chemistry of *Lactobacillus acidophilus*, Crowder & Anderson (53) examined the composition of the neutral fat fraction. They isolated cholesterol, glycerol, lauric, myristic, palmitic, stearic, and oleic acids together with a small amount of a mixture of liquid saturated fatty acids (mean mol. wt. 262) which were optically inactive. The isolation of small amounts of salicylic and phenylacetic acids from the acetone-soluble fat of tubercle bacilli has been reported by Stendal (54).

Stimmel & King (55) described the  $\beta$ -monoglycerides of the even fatty acids from capric to stearic acid, which were prepared via the  $\alpha, \alpha'$ -benzylidene-glycerol according to the method of Bergmann & Carter (56). McElroy & King (57) synthesized a number of mixed triglycerides of the dilaurin series. In order to ascertain the constitution of Schizoneura "wax," Schulz & Becker (58) synthesized the various mixed triglycerides of palmitic and myristic acids without obtaining, however, a substance identical with the natural product. Clarkson & Malkin (59) investigated the phenomenon of triple melting points of the triglycerides from tricaprin to tristearin. There exist three forms of the triglycerides: a stable  $\beta$ -form with inclined chains, a monotropic  $\alpha$ -form with vertical chains, and a glasslike, not truly crystalline,  $\gamma$ -form.

It may not be useless to draw attention to the continuation of the important researches on the metabolism of glycerides<sup>1</sup> undertaken by Verkade & van der Lee (60). The formation of dicarboxylic acids by  $\omega$ -oxidation of the fatty acids, fed in the form of their glycerides, was found to be limited, practically, to tricaprin and triundecylin. After feeding tricaprin, sebacic acid, together with smaller amounts of suberic and adipic acids, was isolated from the urine; from triundecylin, undecanedioic, azelaic, and pimelic acids were obtained. It appears probable that the dicarboxylic acids originally formed give rise to the lower acids by subsequent bilateral  $\beta$ -oxidation.

Hilditch (61) discussed the use of the ester-fractionation method in the analysis of natural fats. Hilditch & Jones (62) described a procedure for ascertaining the distribution of the different glycerides in an unsaturated fat whose acids belong to the  $C_{18}$  series. A method for the gasometric microdetermination of lipids in plasma, blood cells, and tissues was developed by Kirk, Page & van Slyke (63).

#### CONSTITUENTS OF PHOSPHATIDES

Ault & Brown (64) determined the composition of the fatty acids isolated from the phosphatides of beef suprarenals. They obtained palmitic, stearic, arachidic, and possibly myristic acids. The principal unsaturated fatty acids were oleic and arachidonic acids, the latter to the extent of about 22 per cent of the total acids. According to Klenk & Ditt (29), the phosphatides of beef-heart muscle contain 35 per cent solid acids (14 per cent  $C_{16}$ , 21 per cent  $C_{18}$ ) and 65 per cent liquid acids (5 per cent  $C_{16}$ , 45 per cent  $C_{18}$ , 14 per cent  $C_{20}$ , and 1 per cent  $C_{22}$ ), the last two fractions being highly unsaturated.

Klenk (65) examined the chemical composition of the phosphatides of the spleen in a case of Niemann-Pick's disease. The bulk of the phosphatides consisted of sphingomyelin. After hydrolysis, lignoceric and, perhaps, palmitic, stearic, and nervonic acids were obtained.

Kurtz, Jamieson & Holm (66) studied the fatty acids of the lecithin-cephalin fraction of milk. Of the total acids 5.2 per cent consisted of myristic, 16.1 per cent of stearic, 1.8 per cent of arachidic, 70.6 per cent of oleic acids, and 6.3 per cent of a highly unsaturated  $C_{22}$  acid. Another report on milk phosphatides was published by Diemair, Bleyer & Ott (67).

According to Rae (68), the glycerophosphoric acid of egg lecithin

<sup>1</sup> Cf. also this volume, pp. 24, 87, 214, 264. (EDITOR.)

is mainly the  $\beta$ -form, that of liver lecithin consists of about equal amounts of the  $\alpha$ - and  $\beta$ -forms; on the other hand, brain lecithin, brain cephalin, and calcium phosphatide contain mainly the  $\alpha$ -form. The hydrolysis of natural and synthetic phosphatides by enzymes was investigated by King (69). Channon & Foster (70) described the isolation of phosphatides from the wheat germ.

The composition of the phosphatide fraction of *Lactobacillus acidophilus* was determined by Crowder & Anderson (71). The substance yielded on hydrolysis a polysaccharide (over 20 per cent), glycerophosphoric acid, choline, and the following fatty acids: palmitic, stearic, a trace of an acid lower than  $C_{16}$ , and a higher saturated acid, probably a tetracosanoic acid. The unsaturated acids belonged to the  $C_{16}$  and  $C_{18}$  series and could be hydrogenated to palmitic and stearic acids respectively. The polysaccharide, as obtained by saponification of the phosphatide, contained about 9.5 per cent of phosphorus which was split off on heating under pressure with dilute ammonia. The phosphorus-free polysaccharide crystallized in prisms melting between  $160^\circ$  and  $170^\circ$  and showed mutarotation with a final specific rotation of  $+72^\circ$ . By hydrolysis, this apparently new carbohydrate was converted into *d*-galactose and, most probably, glucose and fructose.

#### CONSTITUENTS OF WAXES

A number of very important articles on the constitution of natural waxes have been published by Chibnall and associates. The principal component of the wax from lucerne leaves (72) was found to be *n*-triacontanol (m.p.  $86.3$  to  $86.5^\circ$ ). From this alcohol, *n*-triacontane (m.p.  $65.6$  to  $65.8^\circ$ ) was obtained by reduction, and *n*-triacontanoic acid (m.p.  $93.6$  to  $93.9^\circ$ ) by oxidation. Fatty acids and a complex mixture of paraffins (m.p.  $65.6^\circ$ ) were also present, but no ketones. Wheat wax (73) contains *n*-octacosanol (m.p.  $83.2$  to  $83.4^\circ$ ) as the principal constituent. From this compound *n*-octacosane (m.p.  $61.3$  to  $61.5^\circ$ ) and *n*-octacosanoic acid (m.p.  $90.8$  to  $91.1^\circ$ ) were prepared. Chibnall *et al.* (74) showed in a very thorough investigation that coccerin, the wax from the cochineal insect (*Coccus cacti*), is composed of the following constituents: 15-keto-*n*-tetratriacontanol,  $C_{34}H_{68}O_2$  (m.p.  $100.5$  to  $100.7^\circ$ ),  $CH_3 \cdot (CH_2)_{18} \cdot CO \cdot (CH_2)_{13} \cdot CH_2OH$ ; 13-keto-*n*-dotriacontanoic acid,  $C_{32}H_{62}O_3$  (m.p.  $104.5$  to  $105^\circ$ ),  $CH_3 \cdot (CH_2)_{18} \cdot CO \cdot (CH_2)_{11} \cdot COOH$ ; and *n*-triacontanoic acid,  $C_{30}H_{60}O_2$ .



Betrabet & Chakravarti (75) reported the occurrence of cerotic acid and carnaubyl alcohol in the wax from alkanet root (*Anchusa tinctoria*).

According to Schulz & Becker (76), "Schizoneura acid" obtained by saponification of Schizoneura "wax" consists of a mixture of two parts of myristic and one part of palmitic acids. This "wax" is, in reality, a triglyceride. The composition of the fatty acids of Chinese wax was studied by Koyama (77). Kraut *et al.* (78) investigated the enzymes of the wax-moth (*Galleria mellonella*) larvae, but were not able to obtain a wax-splitting enzyme. Stendal (79) reported in the wax of tubercle bacilli the presence of a glycol, "phthyglycol,"  $C_{26}H_{54}O_2$  (m.p.  $73^\circ$ ;  $[\alpha]_D = -4.2^\circ$ ).

#### INDIVIDUAL FATTY ACIDS

Channon, Irving & Smith (80) reported the results of a very careful investigation of the octadecenoic acids of pig liver. This problem is of interest in view of Hartley's (81) finding of 12-octadecenoic acid in this organ and the theory based thereon concerning the desaturating function of the liver. Channon and his colleagues were unable to confirm these results. They showed that at least 85 per cent of the octadecenoic acids of pig liver consist of the  $\Delta$ -9:10 compound, the dihydroxy-derivative of which can be oxidized to octanoic and suberic acids. There was a second octadecenoic acid present in small amounts which could be converted into a dihydroxy-acid melting at  $117$  to  $120^\circ$  and probably was the  $\Delta$ -10:11 compound.

A spectroscopic study of eleostearic acids from Chinese wood oil was reported by Dingwall & Thomson (82). Maruyama (83) determined the configuration of  $\beta$ -linoleo-tetrabromostearic acid. The constants and properties of pure arachidonic acid,  $C_{20}H_{32}O_2$ , prepared from the phosphatides of beef suprarenals, were described by Ault & Brown (84).

Spielman (85) found tuberculostearic acid, originally isolated from the acetone-soluble fat of tubercle bacilli by Anderson & Chargaff (86), to possess the formula  $C_{19}H_{35}O_2$ . After oxidation with chromic acid in glacial acetic acid solution, small amounts of azelaic acid and methyl-*n*-octylketone were obtained. The formula of tuberculostearic acid therefore is that of a 10-methylstearic acid  $[CH_3 \cdot (CH_2)_7 \cdot CH(CH_3) \cdot (CH_2)_8 \cdot COOH]$ . 10-Methylstearic acid was synthesized from 10-ketostearic acid and it was found that the melting points of the amides and tribromoanilides and the densities and refrac-

tive indices of both the natural and the synthetic acid were practically identical. The melting points of the free acids, however, differed considerably. This discrepancy is explained by the assumption that tuberculostearic acid is one of the two possible optical isomerides and not the racemic compound. That no measurable rotation was observed may be understood in view of the results obtained by Levene *et al.* (87) with the methyl derivatives of fatty acids.

Noller & Bannerot (88) described a partial synthesis of oleic and elaidic acids starting from  $\omega$ -chloro-nonylaldehyde. 12-Ketostearic acid (m.p.  $81^\circ$ ) was synthesized by Perrotte (89). Robinson (90) prepared *n*-triacontanoic acid,  $C_{30}H_{60}O_2$  (m.p.  $93.5$  to  $94^\circ$ ) via the  $\omega$ -bromoundecate, the ethyl- $\alpha$ -acetylbrassylate, and the 13-keto-*n*-triacontanoic acid. The *n*-triacontanoic acid, thus obtained, and *n*-triacontanol (m.p.  $86.5^\circ$ ) formed by reduction of this acid were identical with products prepared by Chibnall *et al.* (74) from coccerin. Schwenk & Priewe (91) reported on the synthesis of 2,15-dimethylhexadecane-1,16-dicarboxylic acid (m.p.  $64$  to  $68^\circ$ ).

The mechanism of the formation of coordination compounds between desoxycholic acid and fatty acids, the so-called choleic acids, was studied by Chargaff & Abel (92). They found that the introduction of side chains or other substituents into the fatty acids (especially into position  $\alpha$  of the carboxyl group) very considerably changed their coordinative power. The coordination numbers of the branched-chain fatty acids were lower and the resulting choleic acids more unstable than those of the normal fatty acids. In the course of this work the synthesis of  $\alpha$ -methyl-*n*-undecanoic acid (b.p.  $125$  to  $127^\circ$  at 6 mm.) was described. Go & Kratky (93) reported interesting results of a study of the X-ray diagrams of various choleic acids.

The *p*-phenylphenacyl esters of all hexanoic acids were prepared by Wrede & Rothhaas (94) in the course of their work on prodigiosin. The *N*-aminotriazoles of some higher aliphatic acids were synthesized by Vorisek (95), and a series of piperazine derivatives of fatty acids by Pollard *et al.* (96). Niederl & Whitman (97) prepared the hydroxy- and the hydroxymethylphenyl-dihydrochaulmoogric acids; Payne *et al.* (98) reported on various chaulmoogric acid derivatives (substituted amines and amides) of therapeutic interest. The vanillylamides of the normal fatty acids (acetic to stearic acids) were studied by Ford-Moore & Phillips (99).

A series of important investigations on the theory of olefinic acids have been published by Linstead & associates (100). Ashton & Smith

(101) studied the addition of hydrogen bromide to undecenoic acid, ethyl undecenoate, undecenol, and undecenyl acetate.

Numerous papers dealing with the oxidation of fatty acids<sup>2</sup> have been published during 1934. Quastel & Wheatley (102) examined the effect of ascorbic acid on fatty acid oxidation in the liver. The autoxidation of linolenic acid and its esters was studied by Goldschmidt & Freudenberg (103). The autoxidation products consisted of comparatively stable peroxides. Smedley-MacLean & Pearce (104) investigated the oxidation of palmitic acid by means of hydrogen peroxide in the presence of a cupric salt. Ponsford & Smedley-MacLean (105) reported on the oxidation of succinic, glutaric, adipic, suberic, azelaic, and laevulic acids under similar conditions. The catalytic influence of potassium ferricyanide on the oxygen absorption of oleic acid was examined by Chow & Kamerling (106). Witzemann (107) studied the oxidation of  $\alpha$ -crotonic, butyric,  $\alpha$ - and  $\beta$ -hydroxybutyric, caproic, and stearic acids by hydrogen peroxide in the presence of disodium phosphate.

Flaschenträger *et al.* (108) published an article on a new mechanism of biological oxidation of the aliphatic chain. By introduction of a substituent into the  $\alpha$ -position they succeeded in deviating the course of the oxidation.  $\alpha$ -Benzenesulfomethylamino-lauric acid was converted by the dog into  $\alpha$ -benzenesulfomethylamino-adipic acid. This result may be explained by the assumption of primary  $\omega$ -oxidation and subsequent threefold  $\beta$ -oxidation [cf. Verkade & van der Lee (60)].

#### HIGHER ALCOHOLS AND HYDROCARBONS

A number of higher alcohols isolated from natural waxes already have been mentioned in the paragraph on wax constituents.

Davies, Heilbron & Jones (109) synthesized the  $\beta$ -octadecylglyceryl ether, in order to compare it with batyl alcohol from the oils of elasmobranch fish. Batyl alcohol previously had been shown by the same authors to be structurally identical with  $\alpha$ -octadecylglyceryl ether. Determination of mixed melting points of the  $\beta$ -compound and its derivatives with batyl alcohol and comparison of surface-film measurements made it clear that the  $\beta$ -compound differed markedly from batyl alcohol, which, on the other hand, is completely identical with the  $\alpha$ -compound.

<sup>2</sup> Cf. also this volume, pp. 24, 83, 214, 264. (EDITOR.)

For ascaryl alcohol (m.p. 84°) from the fats of *Ascaris megalocephala* and *Ascaris lumbricoides*, Schulz & Becker (110) found the formula  $C_{33}H_{68}O_4$ . It contains two hydroxyl groups and no double bonds.

Schoenheimer & Hilgetag (111) found cetyl alcohol in the feces of humans, dogs, and cats, in human intestinal walls, meconium, and in sterile cysts of the intestine. Cetyl alcohol evidently originates in the body. In view of the results reported by Macht (112), who found cetyl alcohol and cetyl acetate to be powerful laxatives, the former may constitute an endogenous purgative.

The preparation of cetyl sulfonic acid by oxidation of cetyl mercaptan was described by Flaschenträger & Wannschaff (113). Bills & Steel (114) found ceryl alcohol in bent grass (*Agrostis*).

According to El Mahdi & Channon (115) *n*-hexadecane is absorbed from the alimentary tract of the rat to the extent of 50 to 100 mg. per day. From the non-saponifiable portion of the ether extract of the eggs of *Bombyx mori* a saturated paraffin,  $C_{18}H_{38}$ , was isolated by Ongaro (116).

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## THE CHEMISTRY OF THE PROTEINS AND AMINO ACIDS\*

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The amino acids and proteins are constituted of but a small number of chemical groups. Recent advances in physics and in chemistry reveal much regarding the structure, the dimensions, and the properties of these groups. X-ray diffraction studies give the distance between carbon atoms in the hydrocarbon chain and in the benzene ring, and the distances between carbon and oxygen, and carbon and nitrogen atoms in the various configurations that have been studied. Most recently X-ray diffraction studies have been supplemented by electron diffraction studies in the observation of the structure of such characteristic configurations as the benzene ring and the carboxyl group (185, 186, 188). X-ray diffraction studies upon proteins reveal further the relations between atomic groupings in the peptide and hydrocarbon chains. Studies of two other kinds also yield knowledge of spatial relations. Measurements of surface layers yield the average cross section through the hydrocarbon, peptide, and protein chains, and measurements of density the apparent specific and molal volumes. The volumes of the chemical groups, like their linear dimensions, prove to be approximately additive and are consistent with their observed cross section. Moreover, "it has become evident that the entropy and free energy of an organic compound are related to its constitution in a simple additive manner" (181).

*Dimensions of CH<sub>2</sub> and CONH groups.*—Whereas aliphatic  $\alpha$ -amino acids may be considered as constituted of amino, carboxyl, and methylene groups, the amino and carboxyl groups combine in peptide and protein molecules, with the loss of a molecule of water, to yield the CONH group. The alternation of CONH and RCH groups

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<sup>1</sup> This review was undertaken on the understanding that a complete survey of the literature would not be attempted, but rather "a systematic treatment of such papers as come within fields which together offer perspectives regarding certain aspects of amino acid and protein chemistry." In the development of these perspectives I have been immeasurably aided by those who, for a great many years, have taken part in our seminar, especially by Drs. Scatchard, Kirkwood, Wyman, McMeekin, Edsall, Ferry, Green, Greenstein, and Salter.

constitutes the characteristic repeating pattern of peptides and proteins. It is the length of these groups as a sum which yields the sharpest lines in X-ray diffraction patterns. Its value has been repeatedly observed, is the same regardless of the protein or amino acid radicle, and may be taken (12, 136, 164, 165), as approximately  $3.5 \text{ \AA}$ . It is accounted for by the interatomic distance between carbon and carbon, and between carbon and nitrogen. The distance separating carbon atoms is  $1.54 \text{ \AA}$  in the diamond, in the carboxyl group and in the hydrocarbon chain. In the direction of the chain the distance separating  $\text{CH}_2$  groups is somewhat smaller because of the tetrahedral valence of the carbon atom and may be taken as  $1.54 \sin 109^\circ 28' / 2 = 1.26 \text{ \AA}$ . The distance between carbon and nitrogen is more in doubt. Hendricks (117) gives it as 1.44 and Hengstenberg & Lenel (118) as between 1.4 and  $1.5 \text{ \AA}$  in their study on glycine, whereas Astbury (10) takes it as 1.32, as it is in urea and thiourea (159). The distance between carbon and nitrogen in the direction of the chain lies, therefore, between 1.08 and  $1.18 \text{ \AA}$ . The length of the spacing,  $-\text{C}-\text{C}-\text{N}-\text{C}-$ , is consistent with these estimates ( $1.26 + 2 \times 1.12 = 3.5$ ) and relates the interatomic distances to the distances between the residues of amino acids in the peptide chains.

This and the other dimensions revealed by X-ray and electron diffraction studies enable us to make accurate estimates not only of the linear dimensions of these groups, but, when related to surface layer and density determinations, of their average cross sections and volumes. The cross section in surface films of aliphatic acids, esters, amides, and urea compounds has been estimated to be  $20.5 \text{ \AA}^2$ , of alcohols to be 20.6, and of acids spread on dilute HCl to be  $25.1 \text{ \AA}^2$  (8, p. 50). The radii corresponding to these diameters are consistent with the volume long since ascribed to the  $\text{CH}_2$  group by Traube (242),

$$V_{\text{CH}_2} = 16.3 \text{ cc.} = 1.26\pi r^2 N = 1.26 \times 21.3 N. \quad (1)$$

The radius corresponding to the cross section,  $21.3 \text{ \AA}^2$ , is  $2.61 \text{ \AA}$ . Studies of homologous series of organic molecules, made for the most part in the last century, are thus entirely consistent with recent physical studies of interatomic and intermolecular distances.

The comparable relation between the length and volume of the amide group yields an estimate of its cross section of  $14.1 \text{ \AA}^2$ . On this basis the average diameter in the neighborhood of the atoms in-

volved in peptide linkage is  $4.24 \text{ \AA}$  (44). In a recent study on protein monolayers, Hughes & Rideal state that  $4 \text{ \AA}$  "is the lesser dimension as determined by means of the X-rays of the cross section of a simple polypeptide chain" (129, p. 62) and Astbury (10) gives  $4.5$  to  $5 \text{ \AA}$  as the distance separating independent or crosslinked parallel peptide chains in proteins.

If the polypeptide chain be considered as spread on a surface with the hydrocarbon chains derived from the amino acids branching from it at distances of  $3.5 \text{ \AA}$ , the peptides of glycine may be considered as simple cylinders of alternating segments of radii  $2.61 \text{ \AA}$  in the neighborhood of the  $\text{CH}_2$  group, and  $2.12 \text{ \AA}$  in the neighborhood of the amide group. The average radius<sup>2</sup> on this basis would be  $2.32 \text{ \AA}$ , which is one-half the distance of nearest approach of parallel polypeptide chains (10, 136).

Substitution of an alanyl for a glycyl residue in the peptide chain may be considered as adding a branch extending  $1.26 \text{ \AA}$  from the cylinder. Valine would extend the branch to  $2.52 \text{ \AA}$ , norvaline or leucine to  $3.78 \text{ \AA}$ , and norleucine to  $5.04 \text{ \AA}$ . On the average the side chain may be considered as extending  $2.52 \text{ \AA}$  from the cylinder or  $4.84$  (i.e.,  $2.52 + 2.32$ ) from its center. In a discussion of the forces of attraction between parallel chains (12, 13, 131), Astbury gives

a "side-spacing" of the order of  $4\frac{1}{2}$  to  $5 \text{ \AA}$  in most of the X-ray photographs of proteins at present available. . . .

The most probable value of the larger of the two side dimensions, that which we have associated with the lateral extension of the side-chains, is not so clear. From existing X-ray photographs it appears to lie generally somewhere between  $9$  and  $10 \text{ \AA}$  [10, p. 199]. The two chief rings [of crystalline pepsin] have spacings of about  $11.5 \text{ \AA}$  and  $4.6 \text{ \AA}$  at ordinary humidity, corresponding to the "side-chain spacing" and the "backbone spacing," respectively, of an extended polypeptide [11, p. 795].

In terms of the dimension of the  $\text{CH}_2$  and  $\text{CONH}$  groups the packing of peptide chains in the two planes should be given by  $4.64$  (i.e.,  $2 \times 2.32$ ) and  $9.68$  (i.e.,  $2 \times 4.84$ )  $\text{ \AA}$ , the amino acids of the protein having hydrocarbon chains, on the average, of the length of valine. The presence of amino acids with longer hydrocarbon chains would, of course, still further increase the distance in this plane between parallel polypeptide chains.

<sup>2</sup> The average radii of the di- and tripeptides of glycine have been estimated as  $2.48$  and  $2.43 \text{ \AA}$  (44).

The cross section of proteins spread in monolayers has been recently extensively investigated. The conditions for spreading even soluble proteins, such as egg albumin, on water have been determined and considered in terms of influence of the structure of the molecule, the number of amino, carboxyl, and amide groups attached to the peptide chain, as well as to the acidity and salinity of solutions ([64], 92, 93, 94, 95, 96, 194). The accumulating evidence indicates that even molecules known to be spherical, when in the body of a solution, assume the dimensions of the polypeptide chain when spread on a surface layer (11, 19, 128, 206). The depth of surface films varies according to Hughes & Rideal (129) up to 12 Å, and according to Fosbinder & Lessig (85) from 10 to 18 Å. The dimensions of proteins exposed to the forces that obtain in the surface layer are thus not very different from those revealed by X-ray studies of the solid protein or of comparable molecules containing the same groups.

*Dimensions of NH<sub>2</sub> and COOH groups.*—The CH<sub>2</sub> group is the least dense element of volume in amino acids, peptides, and proteins, and the carboxyl group the most dense. Recent studies indicate that the characteristic tetrahedral angle is distorted in the carboxyl group.

In the acetate group the angle  $\alpha$  has the value  $124^\circ \pm 3^\circ$ , equal to the value  $125^\circ \pm 5^\circ$  found for formic acid; we may conclude that this value of the bond angle in the carboxyl group is probably retained in all aliphatic acids [188, p. 344].

The carboxyl group attached to the central carbon atom in Figure 1 is drawn to scale from these dimensions. Pauling's studies show no difference between the two oxygen atoms of the carboxyl group, the center of each being 1.29 Å from the center of the carbon atom and their center, extending 0.6 Å beyond that of the carboxyl carbon.

Comparable studies of the equally important amino group have not yet been made. The dimensions of the nitrogen and hydrogen atoms in the figure are those revealed by X-ray and band spectrum data upon comparable compounds, but it remains probable that there is an increase of the angle connecting the nitrogen to the hydrogen atoms, and associated with a decrease in the distance between their center and the center of the nitrogen atom.

The system of apparent atomic volumes developed by Traube (242) from the study of large numbers of organic compounds has recently been shown to be applicable to the amino acids (43, 44, 51). The volumes of the NH<sub>2</sub> and COOH groups as well as of the CH<sub>2</sub> and CONH groups are given in Table I, as well as the corresponding

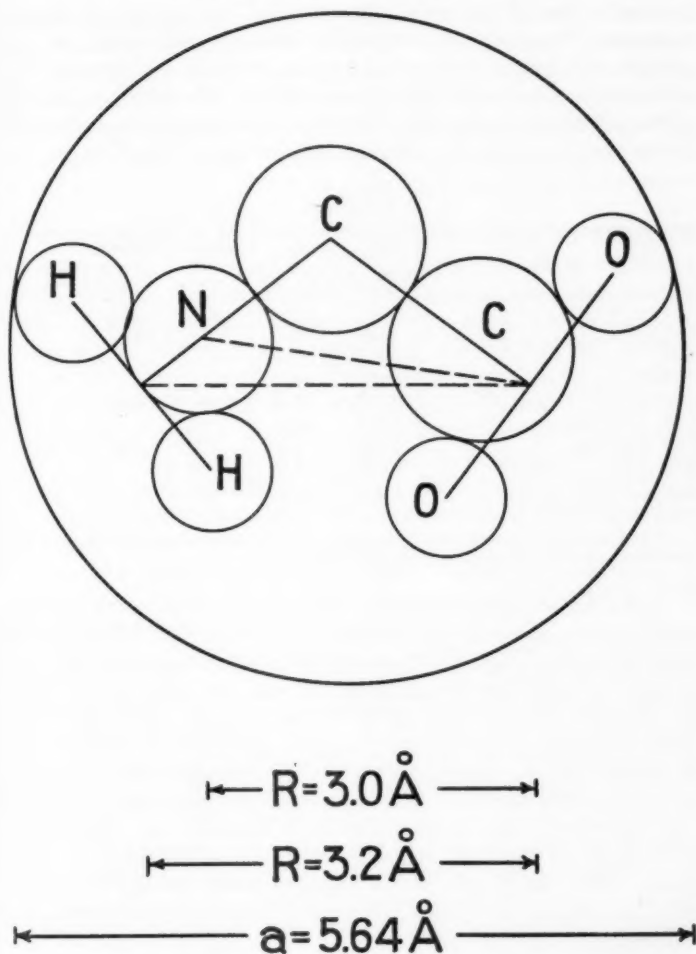


FIG. 1

specific volumes. The partial specific volume of the  $\text{CH}_2$  group is 1.163 as compared with 0.481 for the  $\text{NH}_2$  and 0.420 for the  $\text{COOH}$  groups. The apparent molal volume of their sum,  $\text{NH}_2\text{CH}_2\text{COOH}$ ,

is exactly that of glycine which is one of the densest of organic molecules. The combination in peptide linkage of amino and carboxyl groups, with loss of a molecule of water, increases the apparent specific volume. The sum of the  $\text{CH}_2$  and  $\text{CONH}$ , the alternating groups of the polypeptide chain, is 0.637, which is the upper limit approached by the peptides of glycine that have been studied (Table IV).

TABLE I

Group	Volume of Group		Length in Direction of Chain	Diameter through Chain	Dipole Moment of Group $\times 10^{18} \text{e.s.u.}$
	Per Gram	Per Mol			
	cc.	cc.	$\text{\AA}^\circ$	$\text{\AA}^\circ$	
$\text{CH}_2$ .....	1.163	16.3	1.26	5.22	0
$\text{NH}_2$ .....	0.481	7.7			1.4
$\text{COOH}$ .....	0.420	18.9			1.7
$\text{CONH}$ .....	0.465	20.0	2.34	4.24	3.8
$\text{NH}_2 + \text{COOH}$ .....	0.436	26.6			2.1
$\text{CH}_2 + \text{CONH}$ .....	0.637	36.3	3.5	4.64	3.8
$\text{NH}_2 + \text{CH}_2 + \text{COOH}$ .....	0.572	42.9	4.4*		2.1
$\text{NH}_2 + \text{CONH} + \text{COOH}$ ....	0.448	46.6	5.5		3.2

\* As a cylindrical segment having the diameter of the hydrocarbon chain of 5.22  $\text{\AA}^\circ$ . Considered as a sphere the radius of glycine is 2.82  $\text{\AA}^\circ$ .

The volumes occupied by the amino and carboxyl groups in aliphatic amines and acids are considerably greater than in the amino acids. From studies of the former and of aliphatic amides the volume occupied by these two groups, as a sum, has been estimated as 40.7 as compared with the sum of the atomic volumes, 26.6 cc.<sup>3</sup> The smaller volumes of amino acids have been ascribed to electrostriction of the solvent—the definition of which we shall later consider—and from its amount apparent radii of these groups have been estimated (44), which are in approximate agreement with those calculated from the volumes of the  $\text{NH}_2$  and  $\text{COOH}$  groups, or of ammonia and formic acid. Comparison of the apparent molal volumes of amino acids and betaines (69) yields further information on the approximate dimensions of these groups.

*Electric moments of groups.*—Many of the configurations of which amino acids and proteins are constituted are strongly polar in nature, whereas the  $\text{CH}_2$  group is non-polar. Moreover, the dipole moment of these groups proves to be largely independent of the length of the

<sup>3</sup> The difference is equal approximately to the so-called covolume of Traube.



non-polar hydrocarbon chain to which they are attached. Thus the dipole moment of water is  $1.87 \times 10^{-18}$  e.s.u. When the hydroxyl group is attached to the hydrocarbon chain as in methyl alcohol, the dipole moment is 1.68 and the estimates for all the alcohols thus far studied range only from 1.65 to 1.70. Comparably the dipole moment of ammonia is 1.55, and those of all primary aliphatic amines lie between 1.3 and  $1.4 \times 10^{-18}$  e.s.u. The dipole moments of secondary amines are approximately  $1.0 \times 10^{-18}$  e.s.u.

The dipole moments of the polar groups are not additive as simple numbers but as vectors, and the result depends upon the configuration of the molecule. The values for the carbonyl groups in acetone, methyl ethyl, methyl propyl, and diethyl ketone all range between 2.70 and 2.80, whereas acetyl acetone, containing two oxygens, has a dipole moment of 2.78 (253); those of the carboxyl groups in formic, acetic, and propionic acids are respectively 1.51, 1.73, and 1.72.<sup>4</sup> From certain points of view one is concerned only with the dipole moment of the molecule; from others, the dipole moments of the individual groups (Table I) have significance.

The dipole moment of the amino and carboxyl groups, considered as a sum, is extremely well known from studies upon amino acid ethyl esters dissolved in benzene (260). Neither the length of the hydrocarbon chain nor whether the amino group is in the  $\alpha$ - or  $\beta$ -position appears to influence this value.

The presence of a CONH group, as in glycylglycine ethyl ester, increases the moment only to  $3.2 \times 10^{-18}$  e.s.u. The dipole moment of the amide group alone has, however, been estimated to be 3.2 (261) in the gaseous state and  $3.6$  (60) to  $3.8 \times 10^{-18}$  (144) in non-polar solvents. There is a small effect due to the nature of non-polar solvents (78, 168). Devoto reports the moment of tetraethylurea to be 3.3 and of symmetrical dimethylurea to be  $5.1 \times 10^{-18}$  e.s.u. in benzene at 20° C. The moments of urea and of the amides appear to be higher than can be accounted for as the vector sum of the constituent groups (144), and the heats of formation of these substances have now been shown to be abnormally high (187). It has therefore been suggested that amides resonate between two structures:  $R-C:O(\cdot NH_2)$  and  $R-C \cdot O^-( : NH_2^+)$ . The latter would account for the higher moments. None of these moments is, however, especially large, the mo-

<sup>4</sup> For a discussion of electric moments see Debye (54), Smyth (216), and a general discussion held by the Faraday Society in September, 1934.

ment of 1,8-dinitronaphthalene being far greater, namely  $7.1 \times 10^{-18}$  e.s.u. (121).

*Dielectric constants of solutions.*—The electric moments of molecules have, for the most part, been estimated either in the gaseous state or in non-polar solvents. In biological chemistry we are concerned, however, with behavior in polar solvents. Although there is as yet no completely satisfactory theory for polar solutions, certain relations are apparent between the polarity of molecules and the dielectric constants of solutions.

The greater the density of a molecule and the greater the dipole moments of its groups, the greater the dielectric constant of the solution. Various alcohols all have the same dipole moment and that of water is only very slightly greater. The higher dielectric constant of water than of the alcohols thus depends upon the smaller volumes of molecules containing fewer  $\text{CH}_2$  groups, for it is the concentration of polar molecules in unit volume of solution which determines the dielectric constant (67, 91, 258). Recently Girard has suggested "that at a fixed temperature the value of  $\epsilon$  is approximately proportional to the number of dipoles in unit mass," where  $\epsilon$  is the dielectric constant of the solution, symbolized by  $D$  in this paper, and  $M$  the molecular weight. For the alcohols at  $20^\circ \text{C}$ . we have (91):

TABLE II

	$D$	$D \times M$	$\frac{D \times M}{\rho}$
Water .....	80.4	1448	1450
Methyl alcohol .....	25.7	1183	1498
Propyl alcohol .....	19.3	1160	1444
Butyl alcohol .....	17.0	1260	1556
Amyl alcohol .....	14.5	1275	1560
Octyl alcohol .....	10.0	1300	
Decyl alcohol .....	8.1	1280	1540
Dodecyl alcohol .....	6.8	1265	

If we divide Girard's relation for the higher alcohols by the density of the liquid,  $\rho$ , we refer the dipole moments to unit volume, and the rule that in the absence of complicating circumstances *the dielectric constant varies inversely as the molal volume, when the polarization per mol is the same*, applies not only to the higher alcohols, but also to water.

Formamide and urea, the former containing the amide group, the latter the amide plus the amino group, increase the dielectric constant

of water, but this is not true of molecules containing the same polar groups attached to hydrocarbon chains. Acetamide solutions, unlike those of formamide, do not have higher dielectric constants than water.

Aqueous solutions of amino acids and peptides have far higher dielectric constants than those of any other organic molecules, as was indicated by earlier results (89, 247). Hedestrand's investigations (115) not only established this fact, but demonstrated that a simple linear relation exists between the dielectric constant of the solution,  $D$ , and the concentration,  $C$ , in mols per liter of the amino acid.

$$D = D' + \delta C \quad (2)$$

where  $\delta$  is the increment in dielectric constant per mol of solute and  $D'$  is the dielectric constant of the pure solvent. The dielectric constants of urea-water, of alcohol-water, and of ether-alcohol mixtures have been accurately measured at different temperatures (255, 257). Since urea and the amino acids increase the dielectric constant of

TABLE III

	Dielectric Constant Increment		Dipole Distance		Apparent Molal Volume		Electrostriction of Solvent
	Wyman & McMeekin (259)	De-voto (58, 59)	$\sqrt{\frac{\delta^*}{2.3}}$	$[3.17 + 1.26(n_{CH_2} - 1)]$	Calculated $40.7 + 16.3n_{CH_2}$	Observed (44)	
	$\delta$	$\delta$	$R$	$R'$	$V'$	$\Phi$	$E$
Glycine .....	22.6	26	3.17	3.17	57.0	43.5	13.5
$\alpha$ -Alanine .....	23.2	27	3.17	3.17	73.3	60.6	12.7
$\beta$ -Alanine .....	34.6	35	3.97	4.43	73.3	58.9	14.4
$\beta$ -Aminobutyric acid .....	32.4	36	3.97	4.43	89.6	76.4	13.2
$\gamma$ -Aminobutyric acid .....		52	4.75	5.69	89.6		
$\gamma$ -Aminovaleric acid .....	54.8		4.75	5.69	105.9	90.0	15.9
$\delta$ -Aminovaleric acid .....		63	5.23	6.95	105.9		
$\epsilon$ -Aminocaproic acid .....	77.5	73	5.76	8.21	122.2	104.9	17.3
$\zeta$ -Aminoheptonic acid .....		86	6.24	9.47	138.5		

\*  $\delta$  taken as  $23 + 13.3(n_{CH_2} - 1)$ .

water, whereas alcohols and ether decrease it, mixtures can be prepared which are isodielectric with water (228) and consequently this physical property of the solvent can be distinguished from specific chemical properties.

The value of  $\delta$  for urea is approximately 2.8 (257), that of glycine 22.6 (24, 115, 259). Increasing the length of the hydrocarbon chain from glycine to norvaline does not significantly affect  $\delta$ . Devoto (58, 59) reports a higher value of  $\delta$  of approximately 26 for all  $\alpha$ -amino acids including aspartic and glutamic acids. The difference between these results might be considered methodical were it not that in the case of  $\beta$ -alanine Devoto and Wyman & McMeekin (259) agree that  $\delta$  is in the neighborhood of 35, whereas Hedestrand's results for these amino acids are higher (115). The values of  $\delta$  for all  $\beta$ -amino acids thus far studied are of the same order, and are independent of the length of the hydrocarbon chain, but reflect the distance separating the amino from the carboxyl group.

The aliphatic amino acids (58, 259) show a systematic increase in  $\delta$  with increase in the distance between the amino groups and the carboxyl group. The contribution to the dielectric constant of the amino acid synthesized by Kuhn & Giral (140) in which 14  $\text{CH}_2$  groups separate the polar groups, has not yet been reported. Measurements have, however, repeatedly been made on  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -, and  $\epsilon$ -amino acids. The results of different investigators using different methods in the study of different amino acids are all given by the relation:

$$\delta = (D - D')/C = 23 + 13.3 (n_{\text{CH}_2} - 1) \quad (3)$$

where  $n$  is the number of  $\text{CH}_2$  groups between the amino and carboxyl group, and 23 the value of  $\delta$  for an  $\alpha$ -amino acid. An equation of this type may be compared with that giving the distance,  $R'$ , separating an amino from a carboxyl group in the direction of the stretched hydrocarbon chain.

$$R' = R'_{\text{glycine}} + 1.26(n_{\text{CH}_2} - 1) \quad (4)$$

where  $1.26 \text{ \AA}$  is the distance between carbon atoms.

The peptides of glycine (Table V) have also been studied by Wyman & McMeekin (259) and their results demonstrate that the longer the peptide the greater the value of  $\delta$ . Again one may write

$$\delta = 23 + 46 n_{\text{CH}_2\text{CONH}} \quad (5)$$

where 46 is the increment for each amino acid residue in the chain. This relation may also be compared with the spatial equation:

$$R' = R'_{\text{glycine}} + 3.5 n_{\text{CH}_2\text{CONH}} \quad (6)$$

Were we to assume that both in the aliphatic amino acid series, and in the peptide series, the increase in  $\delta$  were proportional to the increase in distance of separation between amino and carboxyl groups, one might divide the increments in  $\delta$  by the increments in length, obtaining 10.6 and 13.1 per angstrom unit for these series. The implication that the polarity of these molecules increases with the distance of separation of the amino and carboxyl groups is, however, contrary to what would be expected, were the high dielectric constants of amino acids and peptides due to the polarity of the chemical groups. For as we have seen the  $\text{CH}_2$  group is non-polar, and there is no difference between the dipole moments of the esters of  $\alpha$ - and  $\beta$ -amino acids.

TABLE IV

	Apparent Molal Volume	Specific Volume	Dielectric Constant Increment	Dipole Distance	
				Estimated from the Relation $\sqrt{\frac{\delta}{2.3}}$	Estimated from Structural Consideration
	$\Phi$	$\frac{1}{\rho}$	$\delta$	$R$	$R'$
Glycine .....	42.8	0.570	22.6	3.17	3.17
Diglycine .....	77.2	0.584	70.6	5.51	6.67
Triglycine .....	113.5	0.600	113.3	7.02	10.17
Tetraglycine .....	152.6	0.62	159.2	8.32	13.67
Pentaglycine .....	191.0	0.63	214.5	9.65	17.17
Hexaglycine .....			234.2	10.09	20.67
Heptaglycine .....			290.0	11.24	24.17
Lysylglutamic acid .....	172.4	0.626	345.0	12.25	$\begin{cases} 14.23 \\ +3.50 \end{cases}$

*Electric moments of amino acids.*—The great influence of amino acids and peptides upon the dielectric constants of solutions is readily accounted for in terms of the hypothesis—first suggested by Bredig & Küster, and developed by Adam and by Bjerrum—that in these molecules a proton has migrated from the carboxyl to the amino group.<sup>5</sup>

<sup>5</sup> In a previous review (37) I have considered the accumulating evidence, for the so-called zwitterion hypothesis, derived from the amphoteric properties of the amino acids.

As a result the former acquires a negative charge, and the latter is transformed into a positively charged ammonium group. Although such zwitterionic molecules bear no net charge when in the neutral condition and are, therefore, not electrolytes, their charged groups give rise to electrostatic forces, which affect solvent molecules much as do strong electrolytes. Moreover, the separation of a proton from the carboxyl group gives rise to an electric moment equal to the distance of separation,  $R$ , multiplied by the elementary charge on the electron,  $4.774 \times 10^{-10}$  e.s.u. The distance in the case of glycine may be estimated from the X-ray measurements already considered. Provided the positive charge is in the center of the nitrogen atom, the distance in glycine is given by the sum of the distances in the direction of the chain:  $\text{N}-\text{C} + \text{C}-\text{C} + \text{C}-\text{O}$ , which equals  $3.0 \text{ \AA}$  (i.e.,  $1.14 + 1.26 + 0.60$ ), the value  $0.60 \text{ \AA}$  being the distance recently determined for the carboxyl oxygens (185, 188). If the locus of the positive charge were in the orbit of the rotating hydrogen atom, this distance would be somewhat greater. The distance to the edge of the nitrogen atom from the center of the oxygen atoms in Figure 1 is  $3.2 \text{ \AA}$ . Kirkwood (138) estimates the dipole distance of glycine as  $3.17 \text{ \AA}$  on the basis of our studies on the solvent action of neutral salts on glycine at low dielectric constants (38), and Scatchard & Prentiss (204) estimate the distance at  $3.1 \text{ \AA}$  on the basis of their freezing point measurements and Kirkwood's theory. The dipole length,  $R$ , in glycine cannot, without change in the valence angle, be appreciably smaller than the distance,  $R'$ , calculated from X-ray data. Diverse evidence thus indicates that the distance separating the positive and negative charges in glycine lies between  $3.0$  and  $3.2 \text{ \AA}$ . Multiplying by the electronic charge yields estimates of  $14.3$  and  $15.3 \times 10^{-18}$  e.s.u. for the dipole moment of glycine. This value is more than double that of the most polar molecule in Smyth's tables (216) and readily accounts for the high dielectric constants of amino acid and peptide solutions.

The positive and negative charges on the surface of most molecules of this type render them insoluble in non-polar solvents. Their dipole moments in benzene have heretofore not been measured. Recently Hooper & Kraus (123) have studied electrolytes containing complex non-polar organic radicals which were soluble in non-polar solvents. They report dipole moments in benzene at  $25^\circ \text{ C.}$  of  $10.7$  and  $19.4 \times 10^{-18}$  e.s.u. for silver perchlorate and tetraisoamyl ammonium picrate.

The theoretical interpretation of the dielectric constant in polar solutions is a difficult problem (54, 67). A modification of the classical theory for the case of polar solvents has recently been suggested by Wyman (258) according to which the polarization,  $p$ , of a strongly polar solution is given by

$$\frac{\epsilon - 1}{3} = p \quad (7)$$

instead of by the Clausius-Mosotti equation. For a system of  $n$  components of total molar polarizations,  $P_1, P_2, \dots, P_n$ , this may be written as

$$\frac{\epsilon - 1}{3} = \frac{C_1 P_1 + C_2 P_2 + \dots + C_n P_n}{1000\rho} \quad (8)$$

where  $\rho$  denotes density and  $C$  concentration in mols per liter. A relation of this kind would make  $\delta$  a nearly linear expression of the polarization of the solute molecules. According to it, and making use of Debye's (54) relation between electric moment and polarization<sup>6</sup>  $\delta$  is linear, not in the dipole moment,  $\mu$ , but in its square. Wyman's theory would, because of the simplifying assumptions involved in its derivation, be expected to yield maximum values for the dipole moments of amino acids. He has made an estimate of  $21.7 \times 10^{-18}$  e.s.u. for the electric moment of  $\alpha$ -aminobutyric acid in water.

If we adopt the notion that  $\delta$  is linear in  $R^2$ , and term the proportionality constant  $\alpha$ , we may write

$$\delta = \alpha R^2 - K \quad (9)$$

where  $K$  is the decrement in dielectric constant due to a non-polar molecule, greater the greater its molal volume. Taking  $\delta$  for glycine as 23, and  $R^2$  as  $10 \text{ \AA}^2$  we have

$$\alpha = 2.3 + K/10 \quad (10)$$

Studies on the benzbetaines (69), regarded as rigid molecules, suggest a somewhat lower value of  $\alpha$ , namely 1.55, but for the present we shall adopt the proportionality constant deduced from the studies upon glycine.

Provided  $K$  is small as compared with  $R^2$  we may tentatively esti-

<sup>6</sup>  $\mu = .0127 \sqrt{(P - P_0) T} \times 10^{-18}$  e.s.u.



mate the dipole distance and moments of amino acids and peptides from the dielectric constants of their solutions and the relation:

$$R = \sqrt{\delta/2.3} \quad (11)$$

Estimates of  $R$  calculated by means of the approximate equation,<sup>†</sup> 11, are given in Table III and compared with those calculated on the theory that the amino acids are rod-shaped molecules and that the distance,  $R'$ , increases by  $1.26 \text{ \AA}$  for each  $\text{CH}_2$  group between the positive and negative charges. This comparison would suggest that amino acids are not rod-shaped molecules, but show considerable curvature in solution. Even greater curvature might have been expected on the basis of the electrostatic attraction between the negatively charged carboxyl and positively charged ammonium groups. When the latter is in the  $\epsilon$ -position one might indeed have expected ring formation to occur, but were this the case, the molecule should have no appreciable dipole moment, or influence upon the dielectric constant of the solution. Whatever curvature occurs is far smaller than this expectation.

Werner Kuhn & Hans Martin (143) have considered the problem of the shape of zwitterions as revealed by dielectric constant measurements, and concluded that

the electric moment, and therefore the distance between the end groups of the molecules increase proportionately with the square root of the number of members in the chain . . . on the basis of statistical considerations [143 (p. 1528), 142].

Devoto (61) has raised certain interesting objections to this treatment.

The probable length of a chain of  $n$  members possessing free rotation around all valence bonds, and given by Kuhn for long chains as proportional to  $n$ , is calculated by Eyring (75) in a study of the resultant electric moment of complex molecules, to be:

$$C_1 [n + Z(n-1) \cos (-) + 2(n-2) \cos^2 (-) \dots + 2 \cos^{n-1} (-)]^{1/2} \quad (12)$$

<sup>†</sup> If  $K$  be neglected for urea, a doubtful procedure,  $R$  is  $1.1 \text{ \AA}$  and  $\mu$   $5.2$ . Devoto (60) finds the dipole moment of tetraethylurea to be  $3.3$  and of symmetrical dimethylurea to be  $5.1 \times 10^{-18}$  e.s.u. in benzene at  $20^\circ \text{ C}$ . Urea, like formamide, has an electric moment suggesting resonance of an electron (187), but not necessarily the shift of a proton. Its moment is not greater than those characteristic of many polar molecules, and the effect upon the dielectric constant of water depends largely upon its small molal volume.

"where  $C_1$  is the distance between neighboring carbon atoms, and where the angles between the successive bonds are zero for a straight chain."

The dielectric constants of amino acids and peptides unquestionably depend upon the distance separating the charged groups. The dielectric constant increment,  $\delta$ , increases with this distance both in the amino acids and in the peptides that have been studied. According to Wyman's hypothesis the dipole moment,  $\mu$ , and therefore the dipole distance,  $R$ , should increase proportionately with the square root of  $\delta$ . According to Werner Kuhn's hypothesis, based upon the probable twisting of a molecule, on the assumption of free rotation at the valence angles of long chains, the average distance,  $R$ , between the charged groups should be proportional to the square root of  $R'$ , the distance between the charged groups in the stretched condition. In amino acids and peptides one might either expect that  $R$  would be less than the square root of  $R'$ , because of steric hindrance, or smaller because of electrostatic attraction between charged groups.

*Electric moments of peptides.*—Peptides of glycine may, as we have seen, be considered as cylinders whose length increases by  $3.5 \text{ \AA}$  for each molecule in the chain. Estimates of  $R'$ , the distance between the charged groups in the stretched condition, are calculated in Table IV. The heptapeptide of glycine should, on this basis, have a length equal approximately to the radius of egg albumin. Dielectric constant measurements on the peptides of glycine, however, indicate considerable twisting of the peptide chain. Estimating the dipole distance by means of equation 11 yields a length less than half that in the stretched condition. One may, therefore, conclude that free rotation occurs approximately to the extent expected by Kuhn's theory.

Lysylglutamic acid, a peptide synthesized by Bergmann, Zervas & Greenstein (17), has a greater effect upon the dielectric constant of water than any peptide thus far studied (108). It is a double dipole bearing two positive and two negative charges in the isoelectric condition. The inner dipole in lysylglutamic acid may be considered glycylglycine, and the vector sum of the two dipoles, estimated from structural considerations, is  $17.75 \text{ \AA}$ . Although this estimate of  $R'$  is smaller than that for the hexa- and heptapeptides of glycine, the dielectric constant increment is greater. Indeed the average dipole length of the molecule is not very much smaller than that estimated from structural considerations.

The two negatively charged carboxyl groups are on the one side

and the two positively charged ammonium groups are on the other of the peptide linkage in lysylglutamic acid. As a result one might expect electrostatic repulsion between the  $\alpha$ - and  $\epsilon$ -amino groups of the lysine and between the carboxyl groups of the glutamic acid residue. These repulsions should lead to such a stretching of the hydrocarbon chains, as has been shown to occur in dicarboxylic acids.<sup>8</sup> Lysylglutamic acid thus appears to remain a rod-shaped molecule in solution by virtue of its charged condition and, though not very long, may well be the prototype of those proteins, such as myosin, which are long rod-shaped molecules and give rise to double refraction of flow (25, 170).

*Dimensions of proteins and dielectric constants of their solutions.*

For the most part X-ray diffraction studies of proteins have been carried out in the dried condition. Bernal & Crowfoot (19) note of crystals of pepsin that

when examined in their mother liquor, they . . . are relatively dense globular bodies, . . . that the arrangement of atoms inside the protein molecule is also of a perfectly definite kind, although without the periodicities characterizing the fibrous proteins. The observations are compatible with oblate spheroidal molecules of diameters about 25 A. and 35 A., arranged in hexagonal nets, which are related to each other by a hexagonal screw-axis. . . . Peptide chains in the ordinary sense may exist only in the more highly condensed or fibrous proteins, while the molecules of the primary soluble proteins may have their constituent parts grouped more symmetrically around a prosthetic nucleus [19, pp. 794-795].

The forces that hold the polypeptide chains in the spherical form characteristic of many proteins lead to fascinating considerations (87) not unrelated to the theory of their denaturation.

These newer X-ray studies suggest protein radii consistent with those calculated from ultracentrifugal (229) and diffusion (145, 160, 176, 240) studies. The molecular weight and volume of crystalline pepsin (174, 175, 195) and trypsin (177) appear to be comparable to those of egg albumin (235), Bence-Jones protein (237), insulin (214), and to muscle hemoglobin (238). The molecular weights of these proteins are all approximately 34,000 to 35,000; the molecular volumes approximate 26,000 cc. per gm. mol. and the radii 22 A°. Svedberg (229, 230) considers Bence-Jones protein an entirely spherical molecule and egg albumin very nearly so. The radius of the latter,

<sup>8</sup> The influence of these effects upon the dissociation constants of amino acids has been considered elsewhere (37) and has since been investigated by Greenstein (104) and by Edsall & Blanchard (68).

estimated by Stokes' law, is  $21.8 \text{ \AA}$ , by Einstein's law  $22.3 \text{ \AA}$ , and estimated as a sphere  $21.7 \text{ \AA}$ . Moreover, the spherical character of this molecule is consistent with other studies (73, 160, 176).

Hemoglobin and serum albumin<sup>9</sup> are not spherical molecules according to Svedberg, but have closely the same molecular weight, volume, and dissymmetry (229). The radius estimated by Einstein's law is close to  $34 \text{ \AA}$ , or considerably larger than the 27 or  $28 \text{ \AA}$  estimated on the assumption of a spherical molecule.

The nature of serum globulin has been much disputed. Sørensen suggested that "the molecular weight of the serum globulins will vary from about 80,000 to about 140,000" (218, p. 56), but Svedberg & Sjögren (236) believed serum globulin to be monodisperse, to have a molecular weight of 103,000, and to be a symmetrical molecule. Adair & Robinson (7) studied the osmotic pressure of the unfractionated globulin of the horse. "In a series of 17 experiments the maximum and minimum values observed were 192,000 and 154,000 respectively." The notion that the various fractions of serum globulin are monodisperse seemed difficult to reconcile with their chemical composition and behavior (30, 72, 154), and von Mutzenbecher & Svedberg (171, 172) now recognize that the globulin fraction is polydisperse.<sup>10</sup> The largest fraction they estimate to have the same molecular weight, 140,000, postulated by Sørensen. The ultracentrifuge reveals a small fraction of still larger molecular weight, approximately 414,000.

Edestin, excelsin, several other plant globulins, and the phycoerythrin of algae, all appear to be nearly spherical molecules, with radii of  $39.5 \text{ \AA}$  (229, 230).

Thyroglobulin, the hemoglobins of several invertebrates, and the hemocyanins appear to be much larger molecules (229, 230). The molecular weight of thyroglobulin has been estimated to be 700,000 (116) and the molecular weights of the red blood proteins of certain worms 2,800,000 (232, 233, 234). Adair & Roche (5) confirm Svedberg in estimating the molecular weights of hemocyanins to be in the

<sup>9</sup> There are three important investigations of the molecular weight of serum albumin by means of osmotic pressure measurements. Adair & Robinson (6, 7) and Burk (29) estimate the molecular weight to be 73,000 and Pauli (182), in a more recent communication, suggests a far lower value, 59,000.

<sup>10</sup> For the most part, it has appeared desirable to consider at this time only such proteins as appear to be monodisperse, and in their so-called "stability range."

millions. Svedberg considers the hemocyanin in the blood of *Helix* to have a molecular weight of 5 millions and a radius of 120 Å (229).

Although the molecular weights of hemocyanin have been estimated to be greater than those of other proteins, the length of the myosin molecule is probably greater.<sup>11</sup> Edsall has estimated that the myosin molecule may be as long as 6,000 Å on the basis of measurements of double refraction (170) and Werner Kuhn's theory (141). This theory cannot be considered final. The tentative calculation suggests that the length of these molecules may be of the order of the anisotropic band in muscle.

The dipole moments of the uncharged organic molecules, thus far measured, do not exceed  $8 \times 10^{-18}$  e.s.u. and those of electrolytes do not exceed  $20 \times 10^{-18}$  e.s.u. Diglycine, the simplest of the peptides, presumably has a larger moment than the salts thus far studied (123) and the moment of the double dipole, lysylglutamic acid, is more than twice as great.

The diameter of even the smaller multipolar proteins such as egg albumin is approximately four times, and of edestin six times, that of the longer dipole of lysylglutamic acid. The difficulties of measuring the electric moments of proteins have, however, not yet been overcome. To the technical difficulties of measuring, and the theoretical difficulties of interpreting, the dielectric constants of solutions of other polar molecules, must be added, in the case of the proteins, the difficulty of employing sufficiently long wave lengths to overcome the anomalous dispersion due to these enormous molecules, and of obtaining solutions of sufficiently low conductivity. Indeed earlier measurements suggested that solutions containing proteins had lower dielectric constants than water. More recently, however, Wyman has shown that zein increases the dielectric constant of 70 per cent propyl alcohol (256), and Marinesco (157, 158) and Errera (23, 71) have reported measurements of the frequency variation of the dielectric constant for dilute aqueous solutions of several proteins including egg albumin, serum albumin, and hemoglobin. Errera estimates very low dielectric constants, between 4 and 5, for these proteins in the solid state, but dielectric constants of their aqueous solutions which yield values of  $\delta$  of between 100,000 and 250,000.

These measurements have recently been discussed by Williams who suggests

<sup>11</sup> An admirable review has recently appeared on the muscle proteins and their relation to the structure of muscle fiber (251).

the possibility of calculating the molecular weights of the several solutes. . . . An analysis of the data reveals that dispersion curves of normal type are obtained for most of the solutions when dielectric constant is plotted as a function of frequency. . . . The molecular weight for hemoglobin calculated from the data of Errera is of the order of magnitude of that reported by Svedberg and Nichols, using ultracentrifugal methods, but the values for egg albumin and serum albumin are high [252, pp. 727-728].

Shutt reports<sup>12</sup> the dielectric constants of aqueous solutions of iso-electric egg albumin measured at 18° C. and a frequency of 110 cycles per second, which yield a value of  $\delta$  of approximately 4,000 (209). Assuming that the dielectric constants of proteins also increase as the square of the mean dipole moment, these measurements suggest a value of  $R$  of 41.7 Å°, or no more than might be expected from a single dipole, although both analytical and physico-chemical (35, 244) studies indicate that egg albumin contains approximately 27 positively and 27 negatively charged groups. The comparable calculation from Errera's measurements (71) yields an estimate of approximately  $1000 \times 10^{-18}$  e.s.u. for the mean electric moment,<sup>13</sup> or approximately that due to five parallel dipoles. These differences indicate that the measurements upon proteins and their interpretation cannot yet be considered satisfactory. Their resultant electric moments will generally be greater than if they were dipoles, but smaller than if all the positively charged ammonium groups were situated as close together as possible and were separated as far as possible from the negatively charged carboxyl groups.

The electric moments of proteins, spread in surface layers, have recently been measured. Such moments presumably depend upon the immersion of polar groups in the aqueous solution and the displacement of water molecules by the polar groups (84, 85, 205). The depth of the monolayers is far smaller than the diameters of the same proteins in the body of a solution, being 9 to 18 Å° according to Fosbinder & Lessig (85), whose data give electric moments per molecule of 17, 39, and  $114 \times 10^{-18}$  e.s.u. for egg albumin, serum albumin, and edestin, respectively. In interpreting these results one must take into account that the moment of such molecules as palmitic acid,

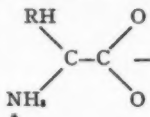
<sup>12</sup> Shutt also discusses the influence of pH on the dielectric constant previously studied by Garreau & Marinesco (90) and by Schulz & Ettisch (207).

<sup>13</sup> Errera, using the Clausius-Mosotti equation, gives the moment as  $44 \times 10^{-18}$  e.s.u., or lower than that demanded by a single dipole having the diameter of the molecule.

spread in a surface film, and presumably due to the carboxyl group, is  $0.16 \times 10^{-18}$  e.s.u. (84, 129), or approximately one-tenth the moment of the carboxyl group estimated in a non-polar solvent. The moments of proteins in the body of a solution are, therefore, unquestionably far greater than when compressed in a surface layer, the latter conditions approaching more closely those characteristic of proteins in membranes or in the solid state.

*Density in the solid state.*—Amino acids and presumably proteins are to be regarded as zwitterions not only in aqueous solution but in the solid state. X-ray diffraction studies upon the crystal structure of amino acids have demonstrated

that the determining factor in the structure of  $\alpha$ -amino acids is the dipole group,



These groups pack together in double molecules or in extended chains.

In glycine the amino groups pack together "so that every  $\text{NH}_3$  is surrounded by six O in the plane of the molecule and by two other pairs above and below" [18 (p. 367), 118]. The result is closer packing than is characteristic of uncharged organic substances, where the distances separating atoms within molecules are far smaller than those between atoms of adjacent molecules (117). "For the simpler amino acids the tendency will be to approach ionic packing (as witnessed by their high specific gravity)" (18, p. 366).

The reciprocals of the specific gravities or densities, the partial specific volumes, of certain amino acids, peptides, and proteins are given in Table V. Those of a few polar molecules such as urea are added for comparison. Despite the absence of the non-polar  $\text{CH}_2$  group in urea, and its effect in increasing the dielectric constant of water, urea is by no means as dense as glycine or the peptides of glycine (Table IV). Glycyl hydantoic acid has the same composition as glycylglycine and *l*-asparagine. The much greater density of the last two thus reflects their zwitterionic structure (161).

The state of amino acids and peptides in the crystal lattice is revealed by studies of the distances, angles of separation, and number of molecules in unit cells. For the most part, Bernal reports four closely packed amino acids as constituting a cell, though cystine forms



cells containing six, and phenylalanine forms cells containing eight molecules.

TABLE V

Substance	Apparent Specific Volume		
	In Solid State	In Aqueous Solution	$\Delta$
Acetamide .....	0.863 (130)	0.931 (65)	+0.068
Propionamide .....	0.960 (130)	0.972 (65)	+0.012
Butyramide .....	0.969 (130)	0.999 (65)	+0.030
Acetylurea .....	0.704 (47)	0.723 (47)	+0.019
Urea .....	0.749 (130)	0.783 (43, 109)	-0.011
Methyl hydantoic acid...	0.715 (161)	0.713 (161)	-0.002
Glycylglycine .....	0.644 (161)	0.584 (161)	-0.060
<i>l</i> -Asparagine .....	0.648 (130)	0.591 (161)	-0.057
Glycine .....	0.622 (46)	0.583 (51, 44)	-0.039
<i>d</i> -Alanine .....	0.714 (46)	0.684 (51, 44)	-0.030
<i>d</i> -Valine .....	0.813 (46)	0.770 (44)	-0.043
<i>l</i> -Leucine .....	0.858 (46)	0.817 (51, 44)	-0.041
Egg albumin .....	0.788 (32)	0.749 (235)	-0.039
Serum albumin .....	0.784 (32)	0.748 (236)	-0.036
Serum globulin .....	0.782 (32)	0.745 (236)	-0.037
Casein .....	0.759 (32)	0.750 (231)	-0.009

*Melting points.*—The greater the forces between molecules in the solid state the higher their melting points. Molecules cannot be said to exist in inorganic crystals where each ion is related to all others surrounding it, and consequently the melting points of salts are higher than those of uncharged molecules. That of sodium chloride is 804°, of potassium chloride, 613° C. The melting point of barium chloride is still higher, 925°, and of lithium iodide, a salt readily soluble in alcohol, lower, 446° C.

The melting points of the amino acids, though lower than those of inorganic salts, are higher than those of most organic compounds. The melting points of amino acids are not accurately known since many decompose when melting, but are in the neighborhood of 300° C., reflecting zwitterionic structure as Pfeiffer (191) and Bjerrum (20) suggested.

Many of the amino acids sublime at temperatures considerably lower than their melting points. The temperatures of sublimation of most of the amino acids have recently been reported, and also the

melting points of the sublimates (28). The latter prove to be very close to the melting points of the amino acids as carried out by improved methods (56, 62). Many of the amino acids do not melt without decomposition. Some give off water, others different decomposition products.

Dunn & Brophy (62) give the melting point of glycine as 289 to 292°, of di- and triglycine as 262 to 265° C. Methyl hydantoic acid, which has the same composition as glycylglycine (161) and which is presumably not a zwitterion, melts almost 100° lower, at 170° C. The melting points of the hydantoic acids are in the same range as those of the sugars; they are higher than that of urea, 133°, and somewhat lower than that of biuret, 193° C., acetyl urea, 215°, or carbonyl diurea, 233° C. These urea derivatives, like hydantoic acid and glycylglycine, all contain the CONH group, and this, we have seen, is a most dense chemical configuration (Table I). Attraction between the CO and NH groups of parallel chains has been suggested as explaining the structure of proteins (10, 22, 131, 132) and such forces might also obtain in the case of smaller molecules containing one or more CONH groups. Molecules containing this configuration all have melting points above 200° C.,<sup>14</sup> but the melting points of the amino acids are still higher, presumably because of close packing due to the charged carboxyl and ammonium groups. Melting points are generally lower if the amino group is not in the  $\alpha$ -position.

*Apparent specific volumes.*—The apparent specific or molal volumes of most organic substances are greater in aqueous solution than in the solid state. This is not true of amino acids or of proteins. Chick & Martin (32) pointed out in 1912 "that there was a shrinkage in volume and corresponding increase in density when" the protein they investigated "formed colloidal solution." In Table V their measurements on the solid state are compared with the recent determinations of Svedberg and his collaborators in dilute solution. Differences between apparent specific volumes in the solid state and in dilute aqueous solution appear to be of the same order for amino acids and for proteins, and opposite in sign to those for most organic molecules (39).

The smaller apparent molal volumes of amino acids and peptides in aqueous solution than in the solid state have been interpreted as due to electrostriction of the solvent (43, 44). The magnitude of this phenomenon, which is of the same order as that characteristic of ions

<sup>14</sup> Oxamide, the diamide of oxalic acid, with a density of 1.667, melts at 419° C.

(246, 248), has been estimated by comparison of amino acids with molecules containing the same chemical groups (44, 250). It increases from 13.3 cc. per mol, when the amino group is in the  $\alpha$ -position, to 17.3 cc. when it is in the  $\epsilon$ -position (Table V), and is 20 cc. when the amino and carboxyl groups are still further separated from each other as on separate molecules. The electrostriction due to lysylglutamic acid, which has two amino and two carboxyl groups widely separated from each other, has been estimated to be 38.8 cc. (108) or slightly less than 10 cc. per charge.

The high density of the proteins cannot be accounted for in terms of the electrostriction of solvent molecules. Egg albumin contains as a maximum approximately 27 free carboxyl and 27 free ammonium groups. The electrostriction of the solvent due to such groups would amount to only a little more than 500 cc. per mol, whereas the apparent molal volume is approximately 26,000 cc. The estimated electrostriction of solvent molecules would, on this basis, be well within the present experimental error in determining the apparent molal volume of this and presumably of most other proteins, though not of multipolar peptides.

The apparent specific volumes of the proteins may be considered as entirely determined by their amino acid compositions. This follows since the apparent molal volumes of the amino acids, unlike most other organic molecules, are approximately given by the sum of the volumes of the groups that they contain. The apparent specific volumes of these groups are given in Table I. If the partial specific volumes of the proteins whose composition is best known, such as gelatin, edestin, and zein, be calculated from the product of the percentage of various amino acid residues, of which the molecule is composed, and the volume occupied by each, estimates of the partial specific volume of the proteins are obtained. It follows from this reasoning that gelatin, which contains a large proportion of glycine and alanine, should have a smaller partial specific volume than zein, which contains a large proportion of leucine. Partial specific volumes of proteins calculated in this way (43) are in as good agreement with those experimentally determined as could be expected on the basis of incomplete knowledge of the amino acid composition of proteins. The characteristic value 0.75 for the partial specific volume of most proteins depends upon the fact that it is very close to the average partial specific volume of amino acid residues.

*Influence of solid state upon solubility.*—The solubility in water of

an uncombined substance is an important constant, characterizing and quantitatively defining certain of its inherent properties. It depends, however, not only upon properties of the liquid phase, but also on those of the substances in the solid state. The forces binding molecules in the crystal lattice influence solubility as well as the attractive and repulsive forces between solvent and solute. When the energy of separating the molecules of an amino acid from each other in the solid state differs widely from that of separating the molecules of other amino acids, this will be reflected by heats of solution (51, 63, 262) and by solubility measurements. Thus, of the two aminobutyric acids, the normal amino acid is the more soluble and has a smaller density and larger molal volume in the solid state. *dl*-Norleucine is also more soluble and less dense in the solid state than *dl*-leucine (46).

Optically active valine and leucine<sup>15</sup> are appreciably more soluble than the racemic forms (46), and among the various isomers of cystine that have been studied by du Vigneaud & Loring (155, 245) those that are optically active prove the least dense in the solid state and the more soluble in water. True, Dalton & Schmidt (51) report higher solubilities for racemic aspartic and glutamic acids than for their optically active isomers, and they raise the question as to whether they are dealing with racemic mixtures or racemic compounds, but with the exception of glutamic acid the *dl*-amino acids appear to have greater heats of solution than their optically active isomers (51, 155, 262).

The extent to which the difference in solubility of stereoisomers depends upon forces between solvent and solute molecules, may be tested by studying solubility in more than one solvent. The ratio of solubility in two solvents should be independent of the crystal-lattice energy, since the solid phase is in equilibrium with both. In so far as the solubility ratio is the same in two solvents one may conclude that the higher solubility of optically active isomers depends upon the fact that their molecules pack less closely in the crystal than do the racemic forms. The ratios in Table VI indicate that this is the case for valine and leucine and suggest a possible secondary effect.

Another approach to this problem is the comparison of heats of solution and heats of dilution. Extensive thermodynamic data regarding the amino acids<sup>16</sup> have recently been reported by Dalton &

<sup>15</sup> The racemic alanine studied by Dalton & Schmidt (51) was slightly more soluble at high temperatures than the *d*-alanine they studied.

<sup>16</sup> Recently the heat capacities, entropies, and free energies of amino acids have also been studied (26, 127, 181).

Schmidt (51) and Zittle & Schmidt (262). Their interpretation demands accurate knowledge of the activity coefficients of the amino acids. Outstanding among their results is their calculation of a negative temperature coefficient for the heat of solution of glycine. The heats of all other  $\alpha$ -amino acids they calculate to be positive, that of valine being greater than that of either *l*- or *dl*-alanine.

TABLE VI

	Radius in Solution (44)	Molal Volume in Solid State (46)	Solubility		$C_0/C_{0.8A}$
			In Water (46, 51, 122)	In 80 % Ethanol (46, 122)	
Glycine .....	b	$\Phi$	$C_0$	$C_{0.8A}$	103.8
<i>d</i> -Alanine .....	3.08	63.6	1.672		
<i>dl</i> -Alanine ....	3.08	62.5	1.656	0.0359	45.9
<i>d</i> -Valine .....	3.46	95.2	0.706	0.0373	18.9
<i>dl</i> -Valine .....	3.46	89.0	0.571	0.0280	20.4
<i>l</i> -Leucine .....	3.63	112.6	0.171	0.0204	8.4
<i>dl</i> -Leucine ....	3.63	110.1	0.0744	0.00848	8.8

*The hydrocarbon chain.*—Solubility in water of the series from glycine to leucine falls off as the number of  $\text{CH}_2$  groups in the molecule increases. Glycine is nearly seventeen times as soluble as *l*-leucine in water, and has approximately the same solubility in 80 per cent alcohol and one-third the solubility in absolute alcohol. In strongly alcoholic solutions the solubility of each amino acid of this series is extremely small, of the same order (46), and tends to increase with increase in length of the hydrocarbon chain.

The great differences between the solubilities of the amino acids in water may, for convenience, be thought of as a summation of two effects: attraction between water and the strongly polar  $-\text{NH}_3^+$  and  $-\text{COO}^-$  groups, and repulsion between water and the paraffin chains.

For their separation, the importance of quantitative information concerning the solubilities of amino acids and their compounds in water and alcohol-water mixtures—employed respectively as their solvent and precipitant since the earliest times—has led to extensive

investigations in three-component systems. Since the affinity of the solute is far greater for the one than for the other solvent, a redistribution of solvent molecules may take place in the neighborhood of solute molecules so that the solvent as a whole can no longer be considered a uniform medium. This probably explains the observation that in systems containing small amounts of alcohol the precipitating action upon amino acids is roughly the same regardless of the length of the terminal hydrocarbon chain. One may contrast the solvent action of very small amounts of alcohol upon the compounds of the amino acids, whose zwitterionic nature has been destroyed, which is greater the longer the hydrocarbon chain. Further, in systems rich in alcohol, change in alcohol concentration has very little further solvent action on amino acid compounds, most of which are more soluble in alcohol than in water, but the precipitating action on amino acids is greater the smaller the hydrocarbon chain and the greater the alcohol concentration. The ratio of zwitterions to uncharged molecules is, of course, decreasing significantly under these conditions, but this should increase rather than decrease solubility in alcohol (66, 68).

Glycine is precipitated by alcohol much as is a uniunivalent electrolyte, and the tetrapole cystine much as is a salt of higher valence, such as barium iodate. The still greater precipitating action of alcohol on such water-soluble proteins as albumins and hemoglobin seems to depend upon their multipolar nature.

Although asparagine and leucine have the same electric moment as glycine, their solubility curves in alcohol-water mixtures reflect, respectively, the amide group and the hydrocarbon chain. The values for the solubilities of asparagine, glycine, and leucine in 20 per cent alcohol are respectively 0.40, 0.47, and 0.57 of those in water. Solubility ratios between water and 80 per cent alcohol are given in Table VI. Those between water and absolute alcohol are still greater (46). Norleucine, with the longest hydrocarbon chain, has the smallest value for the ratio  $C^O/C^A$ , but it is eighty-three times as soluble in water as in alcohol, whereas urea is only ten times as soluble in water as in alcohol, and formylleucine is nearly ten times as soluble in alcohol as in water. Whereas glycine is precipitated by alcohol much as is a neutral salt, formylleucine is dissolved by alcohol much as is the characteristic uncharged organic molecule benzamide, a difference in behavior which must be ascribed to the zwitterionic structure of the amino acid.

The maximum solubilities of the formyl compounds and of the

hydantoins of  $\alpha$ -aminobutyric acid and of leucine occur in 60 and 80 per cent alcohol. Indeed, there is such a balance between the polar and non-polar groups, of which the molecule of the hydantoin of  $\alpha$ -aminobutyric acid is constituted, that it is approximately three times as soluble in 60 per cent alcohol as in either pure solvent, a type of behavior suggestive of the prolamines. Although prolamines contain but a small number of  $-\text{NH}_3^+$  and  $-\text{COO}^-$  groups (15, 40, 50, 173) they are constituted largely of alanine, leucine, proline, and glutamine.

If we compare solubility in two pure solvents, not only are influences due to the solid state largely eliminated, but also redistribution of solvent molecules such as appears to occur in mixed solvents. A simple quantitative relation thus obtains regarding the influence of  $\text{CH}_2$  groups on the solubility ratio. Roughly stated this is that *the ratio of the solubility in alcohol to that in water is increased threefold for each  $\text{CH}_2$  group in the hydrocarbon chains terminating in methyl groups*. This rule appears to hold not only for amino acids but for other sufficiently insoluble compounds such as the formyl amino acids, hydantoins, and hydantoic acids (161). In terms of mol fraction it may be written:

$$\log N^A/N^O = K_2 + 0.49 (\text{CH}_2)_n = K_2 + 0.03 V_{\text{CH}_2} \quad (13)$$

where  $n$  is the number and  $V$  the volume of  $\text{CH}_2$  groups, and  $K_2$  a constant depending upon the nature of the polar groups. The coefficient defining the influence upon the solubility ratio of  $\text{CH}_2$  groups varies, of course, with the pure solvents. A comparable coefficient obtains for the  $\text{CONH}$  group.

When solubility is sufficiently low, solubility ratios yield activity coefficients, related not only to osmotic coefficients and to lowering of the freezing point, but also to the lowering of surface tension. "Willard Gibbs proved thermodynamically that there is a general relation between the surface absorption, the lowering of the surface tension, and the concentration of the solution" (147, p. 147), and Langmuir's studies of surface chemistry are based on these considerations and "are completely in accord with the general relationships found by J. Traube" (147, p. 164) in 1891 (241). "Traube found that with molecules of aliphatic compounds having different lengths of hydrocarbon chains" the decrease in the surface tension of the pure liquid, divided by the partial osmotic pressure of the dissolved substance in the underlying solution, "for dilute solutions increases about threefold for each  $\text{CH}_2$ " (147, p. 164).



*Influence of polar groups and of zwitterionic structure.—*

A comparison of various insoluble organic substances has proved that the spreading tendency depends upon the presence of certain active groups or radicles in the organic molecule, these being the groups which tend to increase the solubility of organic substances in water. For example, pentane,  $C_5H_{12}$ , is practically insoluble in water, but amyl alcohol,  $C_5H_{11}OH$ , is relatively soluble. Thus the hydroxyl groups in organic molecules exert strong attractive forces on the hydroxyl groups in the water molecules and these manifest themselves by an increase in solubility. Similarly the carboxyl group,  $COOH$ , tends to make the lower fatty acids much more soluble in water than the corresponding hydrocarbons [147 (p. 161), 88].

The influence of polar groups upon solubility is quantitatively defined by equation 13. For if we subtract from the solubility ratios of different series of substances the coefficient describing the greater solubility in alcohol than in water due to each  $CH_2$  group, we obtain an estimate for  $K_2$ , the solubility ratio of the terminal polar groups. Thus,  $\log N^A/N^O - 0.03V_{CH_2}$  is  $-3.85$  for all straight chain amino acids,  $-1.12$  for hydantoic acids,  $-1.0$  for hydantoins, and  $-0.84$  for formyl amino acids (46).

The values for all the derivatives of the amino acids differ by more than two, in logarithms, from those of the amino acids. That is to say, the ratios of their solubilities in water to those in alcohol are from 100 to 1000 times as small as those of the amino acids. Such molecules as formylleucine are far more soluble in alcohol than in water, and leucyl amide is soluble even in benzene.

Although the above calculation is satisfactory for the comparison of series differing in the natures of their polar groups and in the lengths of their hydrocarbon chains, it yields no information regarding  $CH_2$  groups situated between polar groups. Do the  $CH_2$  groups in hydantoic acid or in glycine behave like the methyl group in methyl hydantoic acid or in  $\alpha$ -alanine? Allophanic acid is not stable in aqueous solution, nor is carbamic acid. Aspartic and glutamic acids and their amides, asparagine and glutamine,<sup>17</sup> are, however, also  $\alpha$ -amino acids, whose two  $CH_2$  groups are situated between polar groups. Although glutamic acid contains one  $CH_2$  group more than aspartic acid, and glutamine one more than asparagine, the ratio of solubility in alcohol to that in water is not essentially different in either series (161).

<sup>17</sup> The rôle of the amides of dicarboxylic acids in the storage of ammonia has recently been discussed (31, 243).

	Aspartic Acid	Glutamic Acid	Asparagine	Glutamine
Solubility in water: $CO$ .....	0.0375	0.0585	0.186	0.291
Solubility in alcohol: $CA$ .....	0.0000116	0.0000185	0.000023	0.0000315
$CO/CA$ .....	3,233	3,162	8,087	9,328

Such results lead one to conclude that  $CH_2$  groups situated between polar groups appear not to increase the ratio of solubility in alcohol to that in water. Provided the  $CH_2$  groups in asparagine are considered without effect, the very comparable curves for the solubility ratios of glycine and asparagine may be understood. The  $CH_2$  groups in hydantoic acid and in glycine are also situated between polar groups.

The logarithm of the solubility ratio,  $\log N^A/N^O$ , of asparagine is  $-3.402$  and of glutamine  $-3.466$ . Subtracting the value  $-0.631$  for hydantoic acid yields  $-2.77$  and  $-2.83$  as the differences in solubility ratio due to zwitterionic structure. The comparable calculation for glycylglycine yields  $-3.74$  for the difference due to the peptide zwitterion (161).

The dicarboxylic acids and their amides are extremely insoluble both in water and in alcohol. The influence of the  $CONH$  group in decreasing solubility in such series as urea—biuret—carbonyl diurea, acetamide—acetyl urea—acetyl biuret, and urethane—ethyl allophanate, yields quantitative information regarding this effect (47). Glycylglycine, which also contains the  $CONH$  group, is less soluble than glycine, and the higher peptides of glycine are decreasingly soluble in water. Indeed, the solubility of the heptapeptide of glycine is so small in water that it has been studied in a solution of higher dielectric constant (258).

When the space between the positively charged ammonium and the negatively charged carboxyl groups is occupied by non-polar groups the solubilities are far greater than those of  $\alpha$ -amino acids, even when they are packed with equal density in the solid state. The density of  $\beta$ -alanine is 1.404, as compared with 1.401 for  $d$ - $\alpha$ -alanine (51) and 1.424 for  $dl$ - $\alpha$ -alanine. None the less, the solubility of  $\beta$ -alanine in water is more than three times that of the less polar molecules. This effect is particularly striking in the larger amino acids where the influence of the hydrocarbon chain in diminishing solubility is entirely overcome. The densities of  $\alpha$ - and  $\epsilon$ -aminocaproic acids in the solid state are identical, namely 1.174, but the solubility in water of the more polar molecules is 3.84 mols per liter, or over forty times that of

the corresponding  $\alpha$ -amino acid (46). The crystal-lattice energy thus depends upon two factors, the closeness of packing and the magnitude of the dipole moment.

*Electrostatic forces due to zwitterionic structure.*—Born & Fajans in 1920 considered the change in free energy involved in the transfer of ions from an infinitely dilute gas to an infinitely dilute aqueous solution, and formulated an equation which may be written:

$$\bar{F}^0 - \bar{F}_{\text{gas}} = \frac{N\epsilon^2 z^2}{2b} (1/D^0 - 1) \quad (14)$$

in which  $\epsilon$  is the elementary charge of the electron,  $z$  the valence of the ion,  $b$  its radius, and  $N$  Avogadro's number. If the transfer is from water to some medium other than a vacuum which has the dielectric constant,  $D$ , this equation becomes:

$$\bar{F}_e - \bar{F}^0_e = \frac{N\epsilon^2 z^2}{2b} (1/D - 1/D^0) \quad (15)$$

Debye & McAulay (55) employed this equation in their study of mixed solvents, and Scatchard & Kirkwood (203) have extended Debye's treatment to the case of zwitterions. They treat the zwitterion as a molecule made up of two spheres of radius  $b$ , whose centers are separated by a distance  $R$ .

$$\bar{F}_e - \bar{F}^0_e = N\epsilon^2 z^2 (1/b - 1/R) (1/D - 1/D^0) \quad (16)$$

The change in partial free energy of an ion with change in dielectric constant is determined only by the valence of the ion and its radius. The change for a zwitterion is determined not only by these but also by the distance separating the positive and negative charges. The greater this distance, that is to say, the greater the electric moment of the dipole, the larger the change in activity coefficient for a given change in dielectric constant.

Glycine may, as a first approximation, be considered a spherical molecule (Fig. 1), but di- and triglycine have much more nearly the shape of the dumbbell postulated by the Scatchard-Kirkwood equation. Moreover, the members of this series may be compared without introducing any assumption other than that the average radii of their  $\text{NH}_3^+$  and  $-\text{COO}^-$  groups are the same. Assuming that the changes in solubility ratio ascribed above to zwitterionic structure are due to electrostatic forces, and not to chemical interaction with the solvent, equation 16 can be written in the form:

$$\log f_e/(1/D - 1/D^0) = 242.3 (1/b - 1/R) \quad (17)$$

where  $f_e$  is the activity coefficient due to electrostatic forces and the numerical quantity at the right varies only very slightly with the temperature.

The change in solubility ratio ascribed to zwitterionic structure appears to be the same for all  $\alpha$ -amino acids. Estimated by subtracting the logarithm of the solubility ratio of comparable uncharged molecules ( $N_u/N_u^0$ ) from that of zwitterions ( $N_z/N_z^0$ ) we obtain for all monoamino monocarboxylic acids (46):

$$\log f_e = \log N_z/N_z^0 - \log N/N^0 = 3.85 - 1.12 = 2.73 \quad (18)$$

Comparison of hydantoic acid with asparagine yields 2.77 and with glutamine 2.83. These values are in excellent agreement and are lower than the corresponding value for glycylglycine, 3.74 (161). These estimates of the change in free energy due to electrostatic forces render possible estimation of the dipole distance and the radii of the charged groups in terms of equation 17. For glycine we may write:

$$\frac{\log f_e}{242.3 (1/D - 1/D^0)} = \frac{2.77}{242.3 \times 0.02845} = (1/b - 1/R) = 0.40 \quad (19)$$

Solubility studies may thus ultimately give evidence regarding the approximate shape of the molecule.

The Scatchard-Kirkwood equation, because of the assumptions involved in its derivation, can be most advantageously employed in the study of the change in activity coefficients of rod-shaped molecules with change in dielectric constant. Its application, with cautious reservations, not only to longer peptides but to those proteins which show anomalous viscosity and double refraction of flow, should yield interesting results.

Many of the naturally occurring  $\alpha$ -amino acids and most proteins can be regarded more nearly as spherical than as cylindrical molecules. Kirkwood (138) has recently developed a treatment for a model which more closely resembles spherical zwitterions. The molecule is represented by a single sphere, whose radius we shall again call  $b$ , and  $R$  the distance between the charges. If the two charges are equidistant from the center,  $(1/b - 1/R)$  of equation 19 is replaced by:

$$\frac{3R^2}{4b^3} \left\{ 1 + \frac{10}{3} \left( \frac{l}{b} \right)^2 + 7 \left[ \left( \frac{l}{b} \right)^4 - \frac{1}{8} \left( \frac{l}{b} \right)^2 \left( \frac{R}{b} \right)^2 + \frac{1}{96} \left( \frac{R}{b} \right)^4 \right] \right\} \quad (20)$$

where  $l$  is the perpendicular distance from the center of the sphere to the line connecting the two charges, that is, the distance from the center of the sphere to the center of the dipole.

The simplest assumption that can be made for glycine is that the center of the dipole is at or near the center of the molecule (Fig. 1). Under these conditions all terms in the above expression containing  $l$  vanish, and we may solve for  $R$  in terms of  $b$ . Here  $b$  refers to the radius of the glycine molecule, estimated to be  $2.82 \text{ \AA}$  on the basis of molal volume measurements (Table VI). If  $R$  is again assumed to be  $3.17 \text{ \AA}$  the solubility ratio ascribed to electrostatic forces should be 90.3. The agreement with the observed value of 97 may be regarded as extremely satisfactory, since the assumption is made in the above calculation that the center of the molecule coincides with the center of the dipole. This assumption, apparently not quite adequate for glycine, is very far from adequate for such amino acids as alanine, valine, and leucine. For if these branched-chain amino acids be considered spherical the center of the dipole will be farther from the center of the molecule the longer the hydrocarbon chain.

It is probably a better assumption to consider that the charged groups are at an equal distance from the edge of the molecule. The distance from the edge may tentatively be estimated by subtracting from the radius one-half the dipole moment. This yields  $1.23 \text{ \AA}$  (i.e.,  $2.82 - 1.59$ ), a result which may be compared with the radius for the charged groups estimated above as  $1.4 \text{ \AA}$  by the Scatchard-Kirkwood equation.

Kirkwood's theory gives the change in free energy with change in dielectric constant as an

infinite series involving the multipole moments. When the dipole is considerably off center, the higher multipoles are large and the series converges so slowly that it is not sufficient to take only the first two or three terms of the series [139].

Kirkwood has now summed the whole series and obtained an expression which at  $25^\circ \text{ C}$ . becomes:

$$\begin{aligned} \frac{\log f_e}{(1/D - 1/D^0)} = \frac{484.6}{b} \left\{ \frac{1}{(1 - \rho^2/b^2)} - \frac{1}{[(1 - \rho^2/b^2)^2 + R^2/b^2]^{\frac{1}{2}}} \right. \\ \left. + 1.1513 \frac{b^2}{\rho^2} \log_{10} \left[ \left( 1 - \frac{\rho^2}{b^2} \right) \left( 1 + \frac{2\rho^2}{R^2} \left[ \frac{\rho^2}{b^2} \right. \right. \right. \right. \right. \\ \left. \left. \left. + \left( \left[ 1 - \frac{\rho^2}{b^2} \right]^2 + \frac{R^2}{b^2} \right)^{\frac{1}{2}} \right] \right) \right] \right\} \quad (21) \end{aligned}$$

Here,  $q$  is the distance of each charge from the center of the sphere. On the assumption that the radii,  $b$ , of the amino acids are those estimated from molal volumes (44), that the distance of the charged groups from the edge is in all cases  $1.2 \text{ \AA}$ , and that the distance between them,  $R$ , is  $3.2 \text{ \AA}$ , Kirkwood has estimated a value of 96.5 for glycine and slightly lower values for alanine, valine, and leucine (139). The result is gratifying though the assumptions on which the theory and the calculations rest are unquestionably too simple. None the less Kirkwood's equation and calculations offer a model in terms of which one may think concretely about the behavior of amino acids and peptides.

"It is interesting to know what percentage of the total effect arises from multipoles of higher order than the dipole" (139) in Kirkwood's calculations.<sup>18</sup> He estimates that only 14 per cent of the change in solubility ascribable to electrostatic forces is derived from higher multipoles in the case of glycine, 33 per cent for alanine, 52 per cent for valine, and 58 per cent for leucine.

Kirkwood's equation should apply not only to molecules containing one positive and one negative charge but to those containing any number of dipoles situated at certain distances from each other and from the center, or the edge, of a sphere. It should be applicable therefore not only to glycine, but to cystine and even to the multipolar proteins. The solubility of cystine is reduced to a greater extent by alcohol than any other of the naturally occurring amino acids. The effect on proteins, however, is still greater and appears to run parallel with that produced by high concentrations of neutral salts (154).

*Influence of the ionic strength and the electric moment.*—The principle of the ionic strength, accurately formulated by Mellanby in 1905 as a result of his study of the action of neutral salts on globulins, and rediscovered in 1923 by G. N. Lewis as a description of the effect of neutral salts upon each other, acquired theoretical significance in Debye's theory of interionic forces. An important quantity in Debye's theory is  $\chi$ , related to the ionic strength by the equation:

$$\chi^2 = \frac{4\pi e^2}{D K T} \Sigma C z^2 \quad (22)$$

<sup>18</sup> The dipole and multipole moments are defined by equation 14, and the lower multipole moments of a dipole calculated by equation 29, both in Kirkwood's paper (138).

in which  $\Sigma C z^2$  is the ionic concentration, expressed in ions per liter, and designated  $\Gamma$ ; it is approximately twice the ionic strength of Lewis'  $\mu$ , defined as mols per 1000 gm. of water.

The reciprocal of  $\chi$  has the dimensions of length. In the case of a plane electrode

the characteristic length is a measure of the thickness of such an ionic atmosphere (that is the well known Helmholtz double layer). It is . . . a function of the ionic strength, temperature and dielectric constant of the medium [125, p. 216].

Debye's theory of interionic forces has proved adequate to account for the activity coefficients of electrolytes in dilute aqueous solution. It leads to an equation of the form:

$$-\log f_e = \frac{\epsilon^2 z^2}{2.303 \times 2 DKT} \left( \frac{\chi}{1 + \chi a} \right) = \frac{0.5 z^2 (D^0/D) \sqrt{(D^0/D) \Gamma/2}}{1 + a/3 \sqrt{(D^0/D) \Gamma/2}} \quad (23)$$

In dilute solutions of electrolytes the logarithm of the activity coefficient is thus proportional to  $\chi$  and to the square root of the ionic strength. In the case of zwitterions the logarithm of the activity coefficient does not vary as the square root, but as the first power of the concentration. This was empirically discovered by studying the solvent action of neutral salts upon cystine, and theoretically demonstrated by Scatchard & Kirkwood (203), in an extension of the theory of Debye & Hückel to zwitterions. They demonstrated that the term proportional to the square root of the concentration vanishes when the net charge is zero.

The activity coefficients of cystine in the presence of sodium chloride, estimated by the solubility method, are compared with those of sodium chloride in the accompanying figure, so plotted as to demonstrate how completely this zwitterion deviates from the square-root law applicable to ions (Fig. 2). The activity coefficients of most amino acids cannot be determined in water by the solubility method because of their high solubility. The activity coefficients of glycine in sodium-chloride solution have, however, been calculated by Scatchard & Prentiss (204) from freezing point measurements and by Joseph (134) from electromotive force measurements at 1.4° C. in cells with amalgam electrodes, but without liquid junctions. The results of these two



investigations are completely in accord and yield precise information regarding the influence of salt upon glycine and of glycine upon salt.

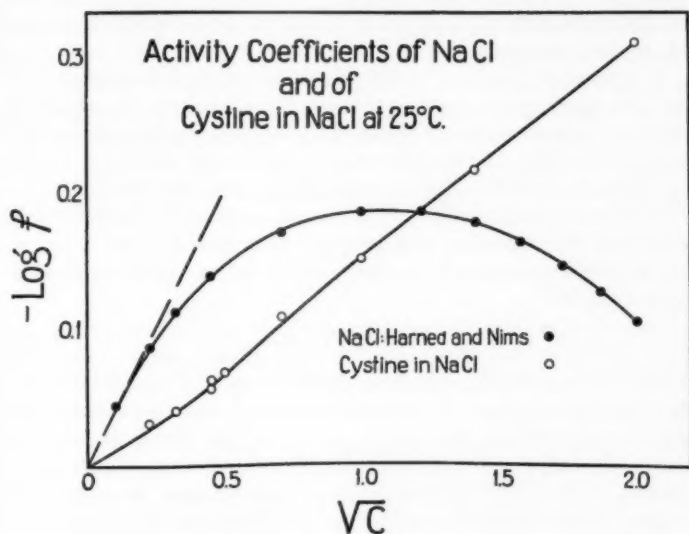


FIG. 2

For ions, the effect of electrostatic forces can be distinguished from that of non-electrical forces since the former varies as the square root of concentration whereas the latter varies as the concentration. This distinction cannot be so readily made for zwitterions because the effect of electrostatic forces is linear with concentration and does not vary as its square root. It follows that the fact that solutions of amino acids and proteins are dilute does not prove that the forces investigated are purely electrostatic in nature.

$\chi^2$  increases not only with increase in concentration, but with decrease in temperature and in dielectric constant (equation 22). Electrostatic forces thus become more important the lower the temperature and the lower the dielectric constant.

The study of the influence of neutral salts upon glycine and leucine in alcohol-water mixtures demonstrated that at sufficiently low dielectric constants the logarithm of the solubility ratio multiplied by the

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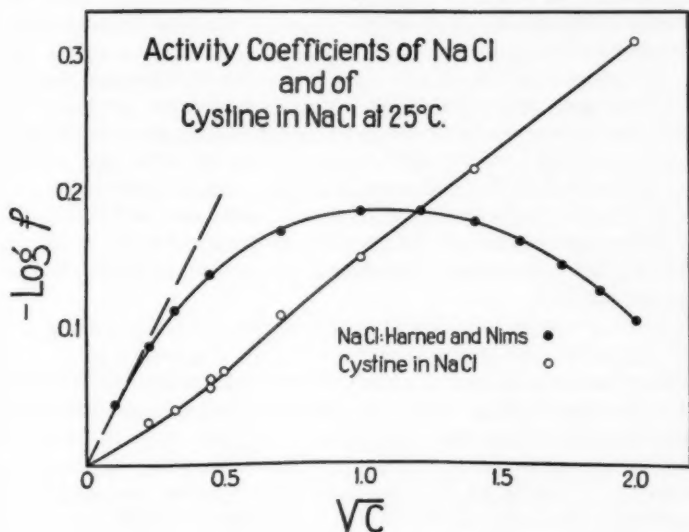


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The study of the influence of neutral salts upon glycine and leucine in alcohol-water mixtures demonstrated that at sufficiently low dielectric constants the logarithm of the solubility ratio multiplied by the

dielectric constant ratio ( $D/D^0 \times \log N/N^0$ ) was a function of  $\chi^2$  (38). So plotted, the results upon glycine in 60, 80, 90, and 95 per cent alcohol all coincide, suggesting that under these conditions the activity coefficients of the amino acids studied were completely ascribable to electrostatic forces.

Scatchard & Kirkwood give an equation for the influence of neutral salts upon amino acids, but Kirkwood's equation for spherical molecules is more nearly applicable to the amino acids thus far investigated (138, 204). Kirkwood's equation gives the logarithm of the activity coefficient of a zwitterion in the presence of a neutral salt as proportional to the ionic strength of the salt, to the square of the dipole moment of the zwitterion, and inversely proportional to the temperature, the dielectric constant, and the sum of the radii of the zwitterion and the salt,  $a$ .

$$a = b_{\text{salt}} + b_{\text{zwitterion}} \quad (24)$$

Taking the radii of salts to be those estimated by Pauling (184), Kirkwood has described our measurements upon glycine in alcohol-water mixtures and deduced the value  $3.17 \text{ \AA}^0$  for the dipole distance of glycine, the value referred to above. Scatchard, using Kirkwood's equation to describe his freezing point measurements, has suggested a slightly lower value for  $R$  of  $3.1 \text{ \AA}^0$ . Although the theory involves many simplifying assumptions and cannot be considered as final, the agreement between these results and those deduced from X-ray analysis of the constituent groups of amino acid molecules must be considered extraordinarily satisfactory.

The solvent action of lithium, sodium, and potassium chloride, and of sodium bromide and iodide upon glycine are identical, within the limits of error, in media of low dielectric constant. The differences in the values of  $a$  (equation 24), estimated from the radii of these salts (184), would be too small to have appreciable effect with  $R^2$  as large as  $10 \text{ \AA}^0$ . Calcium chloride has a slightly greater solvent action at the same ionic strength than the alkali halides, but its radius is not as accurately known. The sulfates are too insoluble to study in alcoholic solution.

The change in free energy of zwitterions with change in ionic strength may, as a first approximation, be considered proportional to  $R^2/a$ . The greater the dipole moment the greater the solvent action of neutral salts. Moreover, if the center of the dipole be considered the center of the molecule, as in glycine and presumably many spheri-

cal proteins, this effect at low ionic strengths will dominate all others, since  $R^2$  will be large in comparison with  $a$ .

The change in free energy is a very complicated function of the ionic strength in the Kirkwood equation, and this is especially true if the dipole is at the edge of the molecule. Under these circumstances the multipole moments cannot be neglected. If the limiting slope alone is considered, all terms up to the octapole moments being included, Kirkwood's expression may be written:

$$\frac{D/D^0 \log f_e}{D^0/D \times \Gamma/2} = -0.125 \frac{R^2}{a} \left\{ 1 + \frac{20}{27} \left( \frac{\rho}{a} \right)^2 \left[ 1 - \frac{R^2}{4\rho^2} \right] + \frac{7}{10} \left( \frac{\rho}{a} \right)^4 \left[ 1 - \frac{5R^2}{8\rho^2} + \frac{5R^4}{48\rho^4} \right] \right\} \quad (25)$$

Assuming the charged groups to be the same distances from the edge as in glycine, and the radii estimated from molal volumes (Table VI), Kirkwood has estimated the salt slopes of  $\alpha$ -amino acids in lithium chloride, taking  $R$  equal to 3.1 and 3.2 Å:

	Glycine	Alanine	Valine	Leucine
	$(D/D^0 \log N/N') / (D^0/D \times \Gamma/2)$			
$R = 3.1$	0.309	0.301	0.291	0.287
$R = 3.2$	0.330	0.321	0.309	0.305

For glycine, only 0.6 per cent of the total effect arises from multipoles of higher order than the dipole. For alanine, valine, and leucine, 4, 10, and 12 per cent arise from higher multipoles (139); these are appreciable effects, but far smaller than those for the change in free energy with change in dielectric constant (equation 21).

The phenomena which must be explained by any theory are that in systems of low dielectric constant the solvent action of neutral salts is nearly the same for all  $\alpha$ -amino acids, though slightly lower for leucine than for glycine (38). The greater value for  $\beta$ -alanine may be ascribed to its higher dipole moment. The solvent action of sodium chloride upon glycylglycine and lysylglutamic acid is still greater. The slopes for glycine, diglycine, triglycine, and lysylglutamic acid, estimated from measurements in 0.05  $N$  sodium chloride in 80 per cent alcohol at 25° C., are 0.32, 0.54, 0.67, and 1.02, respectively. The solvent action upon these peptides at lower ionic strengths, temperatures, or dielectric constants, may of course prove slightly greater. It is interesting to note that these slopes are roughly proportional to

the values of  $R$  estimated from dielectric constant measurements (Table IV). That the ionic strength effect should be approximately proportional to  $R$ , rather than to  $R^2$ , would suggest in terms of Kirkwood's theory that the dipole distances are of the same order as the diameters.

With the exception of glycine these molecules can by no means be considered spherical, but the center of their dipoles may coincide with the center of the molecules. Moreover, their behavior indicates that, as a first approximation, the electrostatic interaction of salts and zwitterions is directly proportional to the dipole moment of the latter, and to the ionic strength of the former, and relatively little affected, in dilute solution, by the size of ions or zwitterions. One may confidently expect that there will presently be available both a larger body of experimental material, and of theoretical calculations, regarding the behavior of zwitterions in regions of low dielectric constant and temperature. For it is under these circumstances that one may expect the ionic strength principle to obtain.

Since the principle of the ionic strength was first deduced from studies upon serum globulin in water at ordinary temperatures, it follows that for these molecules electrostatic forces are dominant, even under these conditions. Although the solvent action of neutral salts upon globulin is a function of the ionic strength (162), it varies with the serum-globulin fraction studied (217).

The solvent action of neutral salts upon certain of the vegetable globulins and upon myosin is far greater than upon serum globulin. Essentially insoluble in the absence of salt, their solubility may increase as much as nineteenfold when the salt concentration is doubled, as is the case of edestin in 0.4 and 0.8  $M$  NaCl (178, 179).

Hemoglobin, more soluble in water, is less influenced by neutral salts but more readily investigated. Cohn & Prentiss (49) described the solvent action on hemoglobin of phosphate, and Green (101) that of chlorides and sulphates, by means of equation 23, the coefficient of the ionic strength being 2.0, or comparable to that characteristic of a bivalent salt. The same relation has been applied to methemoglobin (148).

The globulin recently crystallized by Palmer (180) from the fraction that had previously been considered lactalbumin, has also been studied by the solubility method. Here, too, the logarithm of the solubility appears to vary with the square root of the ionic strength, the proportionality constant being approximately 9. Although the solu-

bility of this molecule is relatively small, 0.3 of a gram per 1000 gm. of water at 30° C., and it has been studied at very low ionic strengths, there is no way of knowing whether the solvent action would not be greater for this globulin (as preliminary measurements indicate it is for hemoglobin) at lower temperatures and dielectric constants.

We have previously suggested that albumins may be regarded as proteins in which the electric moment is large in comparison with size (38).  $\beta$ -Alanine,  $\epsilon$ -aminocaproic, and lysylglutamic acids are so soluble in water that the influence of neutral salts upon them could not readily be investigated by the solubility method. The higher the moment the more rapidly solubility diminishes with diminution in the dielectric constant (equation 20). Although the solubility of  $\beta$ -alanine is still as high as 0.17 gm. per liter in absolute alcohol, the solubility of lysylglutamic acid is reduced to 0.024 gm. per liter in 80 per cent alcohol at 25° C. and of egg albumin to 0.12 gm. per liter in 25 per cent alcohol at -5° C. The precipitation of serum proteins by organic solvents has also been much investigated recently (81, 154, 163).

The solvent action of sodium chloride upon egg albumin has been studied in alcohol-water mixtures at -5° C. (82). Under these conditions denaturation is largely eliminated, and albumins behave much as do globulins in aqueous solution. The logarithm of the solubility ratio of egg albumin also appears to increase as the square root of the ionic strength, the proportionality constant being 2.7, or rather greater than that of hemoglobin in water, though not necessarily in alcohol-water mixtures. The results, plotted as are those in Figure 2, reveal curvature much like that of cystine, but the point of inflection occurs at lower ionic strength.

Scatchard & Kirkwood (203) have deduced the rule that the change in partial free energy of any molecule without a net charge, regardless of its shape or the distribution of its charges, must, at the limit of zero salt concentration, be proportional to the ionic strength and not to its square root. Kirkwood (139) has recently calculated the change in activity coefficient with change in ionic strength of spheres having the dimensions of proteins for various charge distributions. When there are a large number of charges, as in proteins, and their distribution is unknown,

the problem is not entirely determinate, for it is possible that two or more different distributions could lead to approximately the same solubility curve. . . . From the limiting slope alone, no information relative to the symmetry of the



charge distribution can be obtained since it determines only the summed effect of all multipole moments, and leaves their relative magnitudes arbitrary. However, the form of the solubility curve at finite salt concentrations is strongly influenced by the relative magnitudes of the individual multipole moments. . . . We are led to the conclusion that the albumin molecule does not possess an appreciable dipole moment since the dipole solubility curve with the same limiting slope as the experimental curve falls off much too rapidly with increasing salt concentration. On the basis of the foregoing calculations, it seems likely that the charged groups of the albumin zwitterion are so distributed that the dominant multipole moment of the configuration is the quadrupole [139].

The characteristic feature of the curves calculated by Kirkwood is the rapidity with which the slopes fall off with increase in ionic strength. The following slopes for egg albumin, lactoglobulin, and serum globulin at low ionic strengths may be calculated.

TABLE VII

$(D^0/D) C$	$(D/D^0 \log S/S^0) / (D^0/D) C$		
	Hemoglobin (in Water)* (101)	Egg Albumin (in 25% Alcohol) (82)	Lactoglobulin (in Water)* (180)
0.005 .....	22.0	24	132
0.015 .....	12.0	16	68
0.030 .....	8.5	13	48
0.050 .....	6.6	10	37

\* The values of  $S^0$  for these proteins were estimated by extrapolation by use of the square root law.

It is the rapid diminution in slope which accounts for the observation that the logarithm of the solubility of proteins appears to increase with the square root of the ionic strength. The small solvent action at higher ionic strengths would also account for the relatively simple relation which obtains between the logarithm of the solubility and the ionic strength in salt solutions of such concentration that the "salting-out" of proteins is the dominant phenomenon (35).

*Specific salt effects and the Hofmeister series.*—Although leucine is dissolved by salts in a medium of low dielectric constant (38), it is precipitated by the chlorides of lithium, sodium, and potassium from aqueous solution (193). Tyrosine (74, 192) and methionine, also large molecules, are likewise precipitated by sodium chloride,<sup>19</sup> and it has

<sup>19</sup> Leucine preparations often contain methionine, presumably because of their comparable physical properties (167).

been suggested (119) that advantage be taken of this difference in behavior to separate these molecules from amino acids that are either smaller, such as glycine or alanine, or that give rise to larger electrostatic forces, such as cystine.

The solubility of leucine in aqueous sodium chloride is approximately described by the salting-out equation, which applies also to gases (150, 196, 202, 208) and non-electrolytes:

$$\log S = \beta - K_s C = \beta - K_s' \Gamma/2 \quad (26)$$

The electrostatic forces due to the zwitterionic structure of this  $\alpha$ -amino acid are presumably small in a medium of high dielectric constant in comparison with forces due to its long hydrocarbon chain. Its behavior thus suggests the kind of forces which must be considered in addition to those taken into account in the previous section, and which may be expected to be more important the larger the molal volume, the higher the temperature and dielectric constant, and the higher the salt concentration.

Sodium chloride increases the solubility of glycine in water (193), but to a far smaller extent than might be expected from its behavior in alcohol-water mixtures. Moreover, the solvent action of lithium chloride is greater than that of sodium chloride, and that of calcium chloride is still greater at the same ionic strength. Potassium chloride slightly increases the solubility of glycine in dilute, but decreases it in more concentrated aqueous solution.

That salts in concentrated solution have specific effects has been recognized ever since the time of Hofmeister, and that activity coefficients are diminished in dilute, but increased in concentrated, salt solution is characteristic not only of the behavior of amino acids and of proteins, but also of salts. Equations 23 and 25 were derived to describe the decreases in activity coefficients of ions and of zwitterions respectively. Various equations for the increase in activity coefficient<sup>20</sup> have been suggested (53, 55, 126, 202).

Glycine is so soluble in water that the dielectric constant of its saturated solution is as far above that of the pure solvent as is that of water above that of the higher alcohols (Table II). Even were the dielectric constant effect taken into account, activity coefficients could not readily be estimated by the solubility method because of the influences of the large concentrations of zwitterions upon each other.

<sup>20</sup> These equations are given in detail in another place (37).

The influence of zwitterions upon each other is being investigated by studying optically active amino acids in the presence of their racemic mixtures or of proline or hydroxyproline, and by studying such amino acids as cystine, tryptophane, and tyrosine in the presence of other amino acids. The solvent action of amino acids upon each other is greater the greater the dipole moment, and the smaller the molal volume of the amino acid. Whereas glycine increases the solubility of cystine (45),  $\alpha$ -aminobutyric acid decreases it (47). Both the solvent and precipitating action characteristic of salts upon each other, and upon amino acids and proteins, are thus also characteristic of the influence of amino acids upon each other.

Freezing point (86, 124, 149, 190, 201, 204, 224), electromotive force (22, 27, 114, 133, 212), and vapor-pressure measurements (156) can be employed in the study of the effects of amino acids upon each other and upon neutral salts, for the activity coefficients of all components of a system may be thermodynamically related (133) in terms of an equation derived by Bjerrum (21):

$$\frac{\delta \ln f_i}{\delta C_k} = \frac{\delta \ln f_k}{\delta C_i} \quad (27)$$

where  $i$  and  $k$  refer to the several components. The activity coefficients of amino acids and proteins may therefore be calculated from their effects upon neutral salts.

Pauli & Steninger (183) studied the influence of proteins upon insoluble calcium salts, and Failey the influence of a considerable number of amino acids and peptides upon thallous chloride (76) and iodate (77). Stone & Failey (226, 227) have further studied the influence of various proteins upon thallous chloride both as zwitterions and as sodium salts. These studies confirm the theory considered in the previous section, for the influence of the protein salts upon the logarithm of the solubility was found to be proportional to the square root of the concentration, whereas that of zwitterions is linear in relation to the concentration. The slope calculated, per mol of egg albumin, by the Bjerrum equation is approximately 20 at the concentration range studied (near 0.016  $M$   $\text{TiCl}_3$ ), or close to that estimated for the influence of sodium chloride on egg albumin in 25 per cent alcohol at this value of  $(D^0/D) C$  (Table VII). The comparable calculation for hemoglobin is 52 and for edestin nitrate approximately ten times this figure. The activity coefficients of hemoglobin calculated from its influence on thallous chloride are smaller

than those calculated from its influence upon calcium sulphate (183) but considerably greater than that observed in aqueous sodium chloride (Table VII). Unfortunately comparison cannot be made at the same ionic strength and dielectric constant. For the influences of salts upon zwitterions, and of zwitterions upon salts, must yield the same result in completely defined systems.

The studies of Stadie (223, 224) and of Adair (2, 3, 4) should also be related to those considered, but the conditions of the experiments seem too complicated to consider here.

Electrostatic forces do not appear adequate to explain the behavior of amino acids even in dilute aqueous solution. The influence of different salts depends not only upon the ionic strength, but reveals specific effects. These have been investigated by means of the potassium-, sodium-, thallium-, and zinc-amalgam electrodes in cells without liquid junction (133, 134). The ratio of the logarithm of the activity coefficient of glycine to the ionic strength was found to be 0.32 in 0.01 molal  $\text{ZnCl}_2$  at 25° C. and 0.33 at 0° C., results which are in excellent agreement with those calculated from the solvent action of neutral salts in alcohol-water mixtures. The solvent actions of the chlorides and nitrates of bivalent cations have long been known to be very great (193). The influence of alanine, valine, and leucine upon salts reveals progressively smaller values for this ratio, however, the longer the hydrocarbon chain.

The activity coefficients of amino acids in regions of high dielectric constant are decreased more by chlorides and nitrates of bivalent cations than by the alkaline halides, and the solvent action of these diminishes in the order lithium chloride, sodium chloride, potassium chloride. Where the molal volume is sufficiently large, as in leucine, the chlorides of bivalent cations may decrease the activity coefficient, and the alkaline halides increase the activity coefficient, thus decreasing solubility. Acetates, phosphates, and sulphates have, however, greater precipitating action than chlorides, iodates, or nitrates, the effect of the cations remaining in the same order (97, 98, 193).

The so-called Hofmeister series was deduced from studies of the precipitating action of neutral salts upon proteins. The salting-out phenomenon studied by Chick & Martin (33) and by Sørensen (217, 220) was described in terms of equation 26, the concentration of salt being given as mols per liter (35). Since then this equation has been applied to fibrinogen (83), hemoglobin (41, 99, 219), and lactoglobulin (180).

The logarithm of the solubility appears more strictly linear in relation to concentration when the latter is defined as grams per 1000 grams of water than as mols per liter. This is true not only of the salting-out of proteins but of electrolytes (9). There is as yet no theoretical basis for expressing  $K_s$  in terms of molarity rather than molality. Indeed the theory suggests volume-concentration units, which have, moreover, the advantage of convenience. The values of  $K_s'$ , concentration being expressed as  $\Gamma/2$ , are given for the proteins and amino acids thus far adequately investigated in the accompanying table (Table VIII). The salting-out of  $\alpha$ -aminobutyric acid and of leucine has been studied by both the solubility and electromotive force method, yielding satisfactorily concordant results. Only the precipitation of fibrinogen by sodium chloride has been studied, though euglobulin is also precipitated by this salt. The value for hemoglobin is an estimate (101).

TABLE VIII

	NaCl	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Na <sub>2</sub> SO <sub>4</sub>	KH <sub>2</sub> PO <sub>4</sub> +K <sub>2</sub> HPO <sub>4</sub>
	$K_s'$	$K_s'$	$K_s'$	$K_s'$
Cystine (47) .....		0.05		
$\alpha$ -Aminobutyric acid (47, 134)....	0.04			
Leucine (134, 193) .....	0.10			
Lactoglobulin (180) .....			0.6*	
Muscle hemoglobin, horse.....		0.58		
Hemoglobin, horse (99, 101)....	0.12	0.71	0.76	1.00
Hemoglobin, man .....				1.80
Egg albumin (35) .....		1.19		
Pseudoglobulin (35) .....		1.47		
Fibrinogen (83) .....	1.07	1.46		2.16

\* Per 1,000 gm. water,  $\beta$  is 4.13.

Ammonium sulphate has in the past been the most commonly used precipitating agent for proteins, largely because of its great solubility. Thus the solubility of cystine is increased by ammonium sulphate, as by most other salts, up to a value of  $\Gamma/2$  of 4. It is only in the range beyond 7 that equation 26 adequately describes the results. Salting-out is the dominating phenomenon for fibrinogen at ionic strengths greater than 2, for carboxyhemoglobin greater than 4, and for egg albumin greater than 6, under the conditions thus far investigated.

The importance of studying solubility in concentrated salt solutions, not only at a definite ionic strength and temperature, but at definite pH, renders the use of phosphate and acetate buffers par-

ticularly convenient. Since mixtures of known ionic strength, pH, and temperature can readily be prepared (36, 42, 102), the use of phosphate buffers, introduced in the study of hemoglobin (41, 49, 99) and fibrinogen (83), has since been extended to the study of the proteins in serum (30, 146). The analyses of mixtures in terms of salting-out theory readily distinguish fibrinogen, serum globulin, and two serum-albumin fractions [see also Wu (254)]. That serum albumin, though monodisperse (229), yields several crystalline fractions, with different salting-out constants, had previously been demonstrated by Sørensen (218). The value of  $K_s$  observed for pseudoglobulin precipitated from serum (30) is, however, lower than for the pseudoglobulin fraction purified by Sørensen.

The salting-out constant appears to be a characteristic of the protein and to be largely independent of temperature and pH (35). It is of great value in preparation and identification. Though Sørensen has reported a value for carboxyhemoglobin of the horse close to that of Green, obtained at a different temperature and pH, Green has observed a greater value of  $K_s$  for the hemoglobin of man than that previously reported for the horse, and Morgan (also working in this laboratory) a much smaller value for the muscle hemoglobin of the horse, consistent with its smaller molecular weight and volume (238).

The precipitating action of neutral salts upon proteins thus appears to depend upon the charge and the dimensions of the ions, both of the proteins and of the neutral salt. In these terms an explanation may therefore be sought, both of the order in which Hofmeister and subsequent investigators have described the precipitating power of different ions upon the same protein, and of the order in which different proteins are precipitated by the same neutral salt [35, pp. 418-419].

Kirkwood has suggested that the salting-out constants of the larger proteins should be the greater

due to the fact that a zwitterion molecule displaces a certain quantity of solvent and therefore reduces the polarization of the solvent by the salt ion. . . . On the basis of considerations to be discussed elsewhere the following expression for  $K_s$  has been derived:

$$K_s = \frac{4\pi N e^2}{2303 D_0^2 K T} \frac{b^3}{a} \frac{D(D-1)}{2D+1} \quad (28)$$

and indications are that this equation yields a somewhat too large value of  $K_s$  in most cases [139].

The above equation describes an expected effect due to the displacement of solvent molecules by protein molecules, which becomes smaller the lower the temperature and dielectric constant: this, as we

have seen, is consistent with experiment. Even if the above equation is a valid explanation for the polarization effect, and an important factor, it is not necessarily the only factor in salting-out. Equation 28 should be compared with that previously suggested by Scatchard (202).

Whereas  $K_s$  is a characteristic of the protein, the other constant in the salting-out equation,  $\beta$ , reflects its physicochemical state. It varies with temperature and with pH. Its variation with temperature often renders proteins more soluble in concentrated salt solutions at lower than at higher temperature, and permits crystallization by increasing the temperature of concentrated salt solutions saturated with a protein. The change of  $\beta$  with pH has been studied for egg albumin (35, 220), hemoglobin (100), and casein (100, 151, 152, 222). This depends upon the fact that the salts of proteins are more soluble than the neutral molecules, whether in dilute or in concentrated solutions. Analysis of this phenomenon in terms of the solubility-product constants yields estimates of the apparent amphoteric dissociation constants of amino acids and proteins.

*Amphoteric properties.*—Ever since the migration of proteins in an electric field was correctly interpreted as "due to a real electrolytic dissociation at the surface of the particles" (113), the behavior of amino acid and protein ions has become increasingly comprehensible. For this reason the amphoteric properties of amino acids and proteins have thus far not been considered in this review. In sufficiently acid, and in sufficiently alkaline, solutions the chemistry of these substances is not different in principle from that of other ions, although, to be sure, the valence of protein anions and cations is exceptionally high. Anions have a net charge which presumably depends upon the number of free carboxyl groups, and under certain circumstances perhaps also hydroxyl groups, while the net charge of cations depends on the histidine, arginine, and lysine residues in the peptide or protein molecule.<sup>21</sup>

Molecules possessed of a net charge not only give rise to electro-

<sup>21</sup> The very large number of new investigations carried out both by analytical and physicochemical methods, yielding information, in which I have always been much interested, regarding the stoichiometric relations of proteins, have not been reviewed here for lack of space. Electromotive force studies (120, 213) of combining capacity have been supplemented by such different procedures as combination with dyes (50), with hydrochloric acid gas (14, 15, 50) when the protein is in the solid state, and with sodium amalgam (200) when the protein is dissolved in liquid ammonia. Some of these procedures reveal groups other than those that ordinarily dissociate in aqueous solution.



static forces, but are electrolytes. Their movement under a potential gradient has been extensively investigated within the past twenty years, and qualitative observations have given place to quantitative measurements of migration velocity and of the potential between solvent and solute (1, 110, 137, 239).

It is, however, not as ions but as zwitterions that proteins have unique properties. True, the sum of the positive and negative charges, which determine the state of maximum charge, may be greater than the number of charged groups in the zwitterionic condition. It is the distribution of the latter, however, that determines the resultant dipole moment of the molecule, its solubility, the dielectric constants of its solutions, the dissociation constants of its groups, and the electrostriction of the solvent. Many of the properties of the molecule thus depend upon the relation of these groups to its size and shape, and are not greatly influenced by small changes in the reaction of the medium. Thus the salting-out constant,  $K_s$ , appears, within wide limits, to be independent of pH, whereas other properties such as solubility, viscosity, migration velocity, and osmotic pressure, reflect also ionic properties.

The isoelectric and isoionic states (4, 215, 221) have been more scrupulously defined in recent years, and so has the condition of maximum charge (37). This coincides with the isoelectric point only when the maximal acid and base combining capacities are the same. Where this is not the case the condition of maximum charge will occur when not only the zwitterionic but also the excess acid, or basic groups, are dissociated. In certain proteins, such as the albumin and casein of milk, acid groups are in excess; in others, such as gelatin and edestin, the number of basic groups in the molecule predominates.

On the basis of analytical data, casein has half again as many free carboxyl as basic groups, and gelatin twice as many basic as carboxyl groups. In egg albumin and serum albumin the numbers of free acid and basic groups are nearly equal. None the less, all of these proteins have very nearly the same isoelectric point. It is not the number of groups, therefore, but their distribution that determines the isoelectric point. The basic strength of an amino group is diminished by other amino groups and by carboxyl groups, and this is true whether the latter are dissociated or in the undissociated condition.<sup>22</sup> Conversely, carboxyl dissociation is increased by juxtaposition of

<sup>22</sup> Amino acids may be considered to exemplify the former and their esters the latter state (68, 70). See also 37, p. 843.

amino and carboxyl groups, and most other substituents. The result is that the closer amino and carboxyl groups are to each other the more acid the molecule.

The CONH group must also be considered a fairly strong electro-negative substituent. The carboxyl group in asparagine dissociates with a  $pK$  of 2.08, and the amino group of 8.87, as compared with 2.3 and 9.7 for glycine, alanine, valine, and leucine.<sup>23</sup> Consequently, the isoelectric points of these amino acids are in the neighborhood of  $pH$  6, whereas the amide group shifts the isoelectric point in asparagine to 5.5. The isoelectric points of the peptides of glycine are also acid, approaching 5.3 (225).

Among amino acids and peptides, those containing an excess of carboxyl groups are the most acid, and those containing a large number of guanidine groups the most basic. Among proteins, pepsin (174), a globulin, is perhaps the most acid, and the protamines yield a larger proportion of arginine on hydrolysis than any other known protein-like substances (80, 197). The dissociation constants of carefully purified clupein preparations have recently been reported by Rasmussen & Linderstrøm-Lang (198). Their study does not extend to sufficiently alkaline reactions to reveal the dissociation of the guanidine groups, but the other constants are entirely characteristic of peptides. They estimate the molecular weight of clupein to be 4,000, in good agreement with the upper limit indicated by the analytical data of Felix & Dirr (79), but somewhat larger than the ultracentrifugal value of Svedberg. These very basic molecules, studied so much by Kossel and his school, not only have small molecular weights compared to the proteins, but the interesting property of combining with proteins of acid isoelectric points (153). The further study of molecules intermediate in size between the proteins and the peptides may be expected greatly to increase our knowledge of structure and behavior. Among such is crystalline secretin (111, 112), which has a molecular weight of 5,000.

Titration curves have been rendered more readily interpretable by the preparation (16, 17, 106) and study, in the last few years, of a large number of multivalent peptides, in which the charged groups are subject to the influence of known substituent groups and electrostatic forces. The dissociation constants of certain of these are compared below with those of clupein.

<sup>23</sup> The dissociation constants of the amino acids have elsewhere been considered in detail (37, 135, 199).

	$pK_1$	$pK_2$	$pK_3$	$pK_4$
Phenylalanyl-glycine (105) .....	3.10	7.71		
Phenylalanyl-arginine (105) .....	2.66	7.57		12.40
Tyrosyl-arginine (105) .....	2.65	7.39	9.36	11.62
Clupein 1 (198) .....	2.88	7.71		
Clupein 5 (198) .....	3.13	7.92		

Some ten years since, we noted that it was

extraordinary that many of the constants describing the behavior of proteins in relatively alkaline and acid solutions have so nearly the same value. In the titration curves reported  $pK_1$  varied only from 2.8 to 3.7, and  $pK_n$  from 10.2 to 11.1 [35, p. 391].

At that time the titration curve of egg albumin was described in terms of the constants given below. It is interesting that these constants are characteristic of the multivalent peptides that have since been studied (105, 107).

	$pK_1$	$pK_2$	$pK_3$	$pK_4$	$pK_5$	$pKI$
Aspartyl-aspartic acid (103) .....	2.70	3.40	4.70	8.26		3.0
Glycyl- $\alpha$ -amino-tricarballic acid (107) .....	2.70	4.10	5.35	8.32		3.4
Lysyl-glutamic acid (105) .....	2.93		4.47	7.75	10.50	6.1
Egg albumin (35) .....	2.80	4.00	5.65	8.60	10.60	4.8

Titration curves of amino acids and proteins, some in nonaqueous solvents, have since been reported (see footnote 21) and the constants in terms of which the dissociation of the multivalent proteins are described have received a more adequate theoretical interpretation (169, 210, 249). The apparent dissociation constants and the isoelectric points of multivalent peptides and proteins are entirely consistent with each other, indicating that in this respect also the behavior of the proteins is not different in principle from that of smaller molecules containing the same chemical groups.

Dissociation constants are determined in extremely dilute solutions. In more concentrated solutions, activity coefficients must also be considered. The activity coefficients of ions and of zwitterions are, as we have seen, very different functions of the concentration. The still more complicated free energy relations that obtain when the proteins are in states intermediate between the ionic and the zwitterionic have not even been considered in this review.

None the less we have attempted to indicate the forces, known to obtain in amino acid and protein solutions, which must be taken into account in the study of biochemical and physiological problems. Rather than postulate combination, aggregation, or hydration, though

these occur, we have been content to set forth principles in terms of which a complete description of behavior may ultimately emerge. In so doing we have unquestionably laid too much stress on investigations which contribute to the attempted systematic development, and neglected others, perhaps more important, but which appeared to be less readily related in the present state of knowledge.

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## THE CHEMISTRY AND METABOLISM OF THE COMPOUNDS OF SULFUR\*

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It will again be impossible, within the space allotted, to review adequately the numerous papers concerned with the sulfur compounds which have appeared in the last two years. At the risk of excluding many subjects of interest, the review is centered primarily upon the problems of cystine, cysteine, methionine, and their derivatives, since it is in this field that the greatest advances have been made. Certain other aspects will be considered briefly.

### THIOCYANATES

An interesting development has been the revival of interest in the thiocyanates. The non-specific character of the older methods of determination (Munk, Rupp-Schied, etc.) which, applied to urine, may give results far in excess of the amounts actually present (1, 2), has handicapped workers and has led to the development of several modifications (1) or new procedures (2, 3) of greater specificity. In one of these (3), in which the color of the copper-pyridine-thiocyanate complex,  $\text{CuPy}_2(\text{SCN})_2$ , is estimated, the method is said to be accurate for amounts of the order of 5 $\gamma$ .

The normal thiocyanate content of blood, thus determined, was 100 to 200 $\gamma$  per cent (3), a value considerably higher than the value reported for human serum, 30 to 60 $\gamma$  per cent (4). It has been suggested that thiocyanate in saliva may be related to amylase (5), since, in human saliva, the amount present (12 mg. per cent) was much greater than in the saliva of the dog (0.43 mg. per cent), which contains no amylase. Possible differences in cyanogenetic compounds of the diet must be considered, however. The thiocyanate of the gastric juice (dog) appeared to be derived directly from the blood stream rather than from some local source in the gastric mucosa (5). The urinary excretion of thiocyanate in man, in the absence of known dietary sources of cyanogenetic or mustard-oil glycosides, was 0.5 to 3.0 mg. daily (2).

The existence of an enzyme, *rhodanese*, which catalyzes the for-

\* Received January 11, 1935.

mation of thiocyanates from sulfur (or a source of readily available sulfur, e.g.,  $\text{Na}_2\text{S}_2\text{O}_3$ ) and cyanic acid has been demonstrated (6). This enzyme, present in all tissues except blood and muscles, occurred most abundantly in frog and rabbit liver. The existence of such a catalyst was further confirmed by perfusion experiments (7). Negative thiocyanate balances in man were believed to indicate thiocyanate synthesis (7).

In thyroidectomized rabbits (8), conversion of the cyanide group of acetonitrile to thiocyanate, as measured by urinary excretion, dropped from a normal value of 27 to 35 per cent to 3 to 5 per cent. Since thyroidectomy did not influence appreciably the recovery of potassium or benzyl cyanide as urinary thiocyanate, it was concluded that the thyroid catalyzed demethylation of acetonitrile but was not concerned with thiocyanate formation.

#### ERGOTHIONEINE

By a simplified method, similar to that employed for the isolation of ergothioneine from blood, in which the base is precipitated as the cuprous salt (9), the yield of ergothioneine from ergot has been greatly increased (0.18 per cent). This base has also been isolated from normal human urine in quantities of 5 mg. per liter, a figure undoubtedly minimal due to losses in isolation (10). Colorimetric methods indicate much larger amounts (90 mg. per liter), but a considerable portion of this was considered to consist of other substances.

Despite repeated attempts, the synthesis of ergothioneine has not yet been accomplished (11), although the parent amino acid, 2-thiolhistidine, has been prepared (11, 12) and its behavior in the electro-metric titration studied (13). No evidence of basic dissociation of a cyclic nitrogen atom, as in histidine, was obtained. Ergothioneine, thiolhistidine and 2-thiolglyoxaline, but not methionine, were catalysts for the oxidation of cysteine to cystine by hydrogen peroxide in acid solution (14).

#### CYSTINE AND METHIONINE

*Methods of determination.*—The catalytic effect of cystine in buffered solutions of cobaltous chloride on the hydrogen deposition at the dropping mercury cathode has been employed for the microdetermination of cystine in protein hydrolysates (15). The method is stated to be easily and rapidly carried out and to permit the use of small amounts of protein; e.g., a determination is reported in which 0.5 mg.

of human hair was employed. Further studies of the reaction between cysteine and cobalt salts are reported (16). The reaction of cobalt and cysteine has also been made the basis of an indirect micro-method for potassium (17).

In view of the common use of the iodimetric method for the titration of thiol groups, particularly in glutathione determinations, the investigations of this reaction by Lucas & King (18) are important. The iodine consumption of thiol acids was markedly influenced by temperature, theoretical consumption of iodine (i.e., SH to SS) occurring only at or near 0°, while, in solutions distinctly acid, the influence of pH was not a factor. In the titration of cysteine, the concentration of both cysteine and iodine influenced the extent to which oxidation occurred. In the presence of 0.5 per cent potassium iodide, cysteine could be determined accurately by indirect iodimetric titration at 0° and *N* acidity. Cysteine in its reactions with iodine was much more affected by temperature, pH, and dilution factors than was the peptide, glutathione. The iodimetric method has also been modified for the determination of urinary cystine (19) under conditions similar to those found optimal by Lucas & King.

The method of Sullivan has been modified by Lugg (20) in an attempt to secure a better proportionality. It is believed that one difficulty in the original Sullivan procedure lies in the fact that the cystine standard is not highly buffered as is the solution containing cystine in protein hydrolysates. Improvements have been effected by more careful control of pH and by the addition of a considerable amount of amino acid buffer (glycine) to both standard and unknown. Lugg has also examined the sources of error in cystine determinations (21) and has maintained that destruction of cystine (and cysteine) during acid hydrolysis of protein is an important factor, particularly in the presence of carbohydrate. The use of stannous chloride to diminish humin formation is condemned. Since the reduction of cystine by cyanide, used in the Sullivan method, does not yield two molecules of cysteine, it has been suggested that protein hydrolysates be reduced by zinc and hydrochloric acid before the Sullivan reaction is carried out (22). More consistent results are obtained if the cysteine is thus present preformed.

Krijgsman & Bouman (23) have applied photometric measurement of the color of the Sullivan reaction to the determination of cystine in tungstic acid filtrates of blood. The Zeiss photometer has also been used (24) in the measurement of the color produced in the

Lugg modification (20) with pure solutions of cystine and with protein hydrolysates. Further applications of the photometric methods will undoubtedly be of great value. The usefulness of the Sullivan method is recognized, but the many attempts to modify it indicate that, in its present form, the method is not entirely satisfactory in the hands of many investigators.

An indirect gravimetric method for cysteine in proteins (25) is dependent upon the hydrolysis of protein by sulfuric acid in the presence of tin and the precipitation of the cysteine formed as cysteine cuprous mercaptide. The organic sulfur of this precipitate is determined and calculated as cystine. While the values obtained agree well with those obtained by other standard methods, it must be remembered that the formation of an insoluble cuprous complex is a reaction common to many thiol compounds.

That the Folin-Marenzi method may give misleading cystine values is shown by studies on deaminized casein. Steudel and coworkers, using this method, had made the remarkable observation that in deaminized casein, the amount of cystine, as compared with that of casein, was increased. This may be explained by the presence in deaminized casein of sulfur-free derivatives (probably of tyrosine) which give color with the Folin reagent (14). The cystine content of deaminized casein, as determined by the Vickery-White method, was the same as that of the casein from which it was prepared (26).

The existence of cysteine in protein hydrolysates as suggested in Baernstein's preliminary paper has not been supported by the studies of Hess (27), in which both the Sullivan colorimetric and the Okuda iodimetric procedures were used. It should be noted, however, that in the final report of Baernstein, the claim is not made that cysteine or sulfhydryl groups are present, the values being reported as "sulfhydryl and disulfide sulfur."

Further investigations on the determination of methionine in proteins, tissues, and biological fluids are urgent. The only method available, that of Baernstein, has been improved (28). The high methionine content of ovalbumin, as determined by the new modification, is notable (5.24 per cent), the highest content yet reported for any protein. By improved methods of isolation (9), 2.5 per cent of methionine has been obtained from "egg albumin" (presumably not the crystallized protein), and 1.3 per cent from casein. Isolation experiments from other proteins are also reported (29). Hill & Robson (30) have simplified the method of isolation of methionine from protein hydroly-



sates, have determined its isoelectric point (pH, 6.0), and have also presented solubility curves in water and saturated salt solution.

*Alkaline decomposition of cystine.*—The importance of a knowledge of the chemical history of proteins used in nutrition studies, particularly with regard to treatment with alkali, has been emphasized (31). Casein of high purity when subjected to successive precipitations by acid from dilute sodium hydroxide in the manner usually followed in the preparation of pure casein showed a progressive decrease in cystine content from an original figure of 0.336 per cent to 0.033 per cent after 5 reprecipitations. Similarly marked variations have been observed in the cystine content of certain commercial caseins of British origin used for nutrition experiments (22). This variation in the cystine content of casein probably explains the differences in the rate of growth of rats on the same level of casein. The classical results of Osborne & Mendel, in which the action of cystine as a supplement to casein was first observed, were obtained with diets containing 9 per cent of casein. Many recent investigators, in efforts to select a basal level of dietary casein for the demonstration of cystine deficiency and supplementary action, have observed adequate growth on the level of casein used by Osborne & Mendel and it has been necessary to further restrict the amount of dietary casein in the basal cystine-deficient diet.

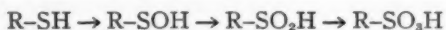
Cysteine was much more stable toward boiling 4 *N* sodium hydroxide than cystine (32). Increasing concentrations of the alkali led to decreasing destruction of cystine. Cystine in crystalline form was isolated in 40 per cent yield from cystine boiled for 12 hours with 4 *N* alkali. This isolated residual cystine decomposed on further treatment with alkali in exactly the same manner as the original cystine. To explain the relative stability of cystine toward alkali, a reaction between some decomposition product of unknown nature and the unchanged cystine, which leads to stabilization of the latter, was postulated. Further studies of the relation between the structure of cystine (cysteine) derivatives and stability toward alkali have included the effect of the substitution of acyl radicals in the amino group (33). Such substitution increased the initial lability of the sulfur, the effect varying with the negativity of the substituent group. Of the disulfides studied, homocystine was decomposed much more slowly than cystine. The presence of a ring structure, as in cystine phenylhydantoin or the dianhydride of dialanycystine, greatly increased the reactivity of the sulfur. Andrews & Andrews (34) were able to identify

as products of the alkaline hydrolysis of cystine phenylhydantoin, sodium sulfide, free sulfur, and 3-phenyl-5-methylene-hydantoin, the latter easily breaking down to give phenylurea and pyruvic acid.

Zahnd & Clarke (35) have extended the studies of the decomposition of cysteine by alkaline plumbite to proteins and protein hydrolysates. The quantitative nature of the reaction leading to the liberation of labile sulfur has been established under conditions suitable for the estimation of small amounts of cystine. The reproducibility of the values obtainable with purified proteins is indicative of the value of such a determination as a source of a new type of analytical data for the characterization of proteins. In most cases, labile-sulfur values approximated the cystine sulfur. "The case of egg albumin, however, and possibly of others points to the possible presence in proteins of components other than cystine or cysteine which yield lead sulfide on heating with alkaline plumbite." The non-labile sulfur of egg albumin was equivalent to the methionine sulfur as obtained by Pirie (9).

*Oxidation-reduction studies.*—The presence of copper salts greatly accelerated the oxidation of cystine in hydrochloric acid solution to cysteic acid by means of free oxygen (36). If the cystine were dissolved in sulfuric or phosphoric acid, no appreciable oxidation occurred either in the presence or absence of copper salts. In experiments designed to confirm the earlier work of Elliott on the proportionality of the rate of oxidation of cysteine to the amount of copper present and to use this reaction as a means of estimation of small amounts of copper, Gad Andresen & Nielsen (37) were unable to obtain the proportionality claimed by Elliott. The latter in reply (38) has maintained that the proper conditions for the reaction were not afforded.

Evidence of the formation of sulfenic and sulfinic acids in the oxidation of cysteine by iodine has been obtained (39), a finding in harmony with the theory that the oxidation of cysteine to cysteic acid involves the gradual addition of oxygen with the formation of a series of intermediate oxidation products,



In the oxidation of cystine in a non-aqueous medium (acetonitrile) by perbenzoic acid, a maximum of 4 oxygen atoms was consumed by each molecule. This suggests the formation of a similar series of oxidation products with the -SS- linkage of the cystine intact. Decomposition of the product of oxidation yielded a mixture of cysteic acid and the

corresponding sulfinic acid (40, 41, 42). More exact knowledge of the conditions for the formation of and the properties of these intermediate oxidation products is important, since similar reactions may play a rôle in the biological oxidation of cystine.

By oxidation of cystine phenylhydantoin with bromine water, the unstable cysteic acid phenylhydantoin-hydrobromide was obtained (34), which was rapidly broken down in aqueous solution to give considerable amounts of inorganic sulfate as one product, a fact which sharply distinguishes it from cysteic and other sulfonic acids and which is possibly of significance in the mechanism of sulfur oxidation.

A new synthesis of taurine from cystinamine in which hydrogen peroxide is used as the oxidizing agent is reported (43). New derivatives of taurine have been prepared (44) and their dissociation constants determined (45). New dipeptides of taurine and cysteic acid with glycine have also been synthesized (46).

The anomalous behavior of cystine in the Van Slyke gasometric method for amino nitrogen has been studied (47). Oxidation of the sulfur of cystine to sulfate has been demonstrated and it is believed that the "extra" nitrogen of the Van Slyke reaction resulted from the reduction of nitrous acid in the oxidation of the sulfur. From the reaction between nitrous acid and thioglycolic acid (a non-nitrogenous thiol compound whose sulfur is also oxidized readily by nitrous acid) a gas was obtained under the conditions of the Van Slyke procedure, which was shown by spectrophotometric methods to be nitrogen. By analogy, the "extra" gas in the cystine-nitrous acid reaction would appear to be nitrogen and not carbon monoxide or oxides of nitrogen as has been suggested.

It has been assumed that the sulfur of cystine in contrast to the sulfur of the thiolimidazole group (as in ergothioneine) is not oxidized by ferric chloride. Recent investigations have shown that cystine but not cysteic acid is readily oxidized by ferric chloride to yield sulfates (48).

When cystine, either in acid (HCl) or alkaline solution (NaOH), was irradiated by unfiltered light from the quartz-mercury lamp (49), free sulfur was observed, hydrogen sulfide was produced, and cysteine was identified in small amounts (5 per cent). This reduction of cystine may explain the peculiar oxidation-reduction system obtained by Wels in irradiation of proteins (50).

*Reactions of cystine and derivatives with monoiodoacetic acid.*—Dickens (51) has prepared the S-carboxymethyl derivatives of cysteine and glutathione by way of the reaction,  $\text{RSH} + \text{ICH}_2\text{COOH} =$

$\text{RSCH}_2\text{COOH} + \text{HI}$ . The reaction of iodoacetic acid with amino groups also took place readily according to the equation,  $\text{RNH}_2 + 2 \text{ICH}_2\text{COONa} + 2 \text{NaOH} = \text{RN}(\text{CH}_2\text{COONa})_2 + 2 \text{NaI} + 2 \text{H}_2\text{O}$  (52). The reaction with SH was more rapid than with  $\text{NH}_2$  groups, so that by avoiding an excess of the reagent, it was possible to obtain the S-ether compound of cysteine with the  $\text{NH}_2$  group unaffected. Not all SH compounds reacted with the same ease as cysteine; glutathione, however, belongs to the rapidly reacting group of SH compounds. The biological significance of these chemical reactions in relation to poisoning by iodoacetic acid has been discussed. The preparation and properties of a series of these carboxymethyl derivatives of cystine, cysteine, and related compounds have been described.

*Isomers and homologues of cystine and methionine.*—The details of the isolation of the internally compensated stereoisomer of cystine, mesocystine, have been described together with the proof of the stereostructure (53). A series of derivatives of the internally compensated mesocystine and the racemic (*dl*) cystine have been prepared and their properties compared. Since all the isomers of cystine were available, it was possible to determine their solubility (54). As anticipated, but in contradiction to certain earlier studies, the solubilities of the *d* and *l* forms were the same. The solubilities of the *dl* and meso forms were also essentially identical and only about half of that of the optically active forms. The fact that *dl*-cystine had a lower solubility than the active forms suggested that *dl*-cystine may be a racemic compound and not a racemic mixture. Some evidence was also obtained of the combination of the *d* or *l* isomer with the meso modification to yield compounds analogous to racemic compounds. The solubilities of stone- and hair-cystine were identical and the solubility of a mixture of the two was unchanged, thus affording good evidence of the identity of these two forms.

The synthesis of the 5-carbon homologue of cystine, bis- $\delta$ -amino- $\delta$ -carboxybutyl-disulfide (*pentocystine*) and of the next higher homologue of methionine,  $\delta$ -methylthiol- $\alpha$ -amino-valeric acid (*homomethionine*), and of various derivatives of these has been described (55).

*Cystine content of proteins.*—Several studies concerned with the question of the constancy of the cystine content of the same protein fraction in the individual or the species have been undertaken. The value of such studies is in part lessened by the inadequacy of the methods of fractionation of proteins. In analyses of glycinin from 10

varieties of soy beans, the cystine content varied between 0.74 and 1.46 per cent (56). It is believed that the differences are probably to be explained by the variable proportions of the different globulins in the glycinin fraction. Species differences in the cystine content of hemoglobins (Vickery-White method) were observed, 0.41, 0.61, and 1.16 per cent of cystine being present in the hemoglobin of the horse, sheep, and dog respectively (57). The sulfur also varied (0.39, 0.73, and 0.57 per cent respectively) but the iron content was uniform. Marked differences in the cystine content (Folin-Marenzi) of the albumin and globulin fractions of human sera were observed (58). In 16 sera, the average cystine content of the albumin fraction was 6.07 per cent (range, 4.82 to 7.40) and of the globulin fraction, 3.64 per cent (range, 2.34 to 4.70). The variation of the cystine content of the total serum proteins was less marked, the limited range of 4.27 to 5.20 per cent suggesting that the greater variations in the individual fractions may be due to inadequate fractionation. In a similar study of the sera of other animals (59), the highest figure for the albumin fraction (8.5 per cent) and lowest for the globulin fraction (2.0) were observed in a specimen of chicken serum.

The cystine content of the proteins of pasturage is important in relation to the problem of dietary cystine versus cystine synthesis for wool production and has been discussed in previous volumes of this *Review*.<sup>1</sup> Pollard & Chibnall (60), employing the methods of Chibnall, have prepared and analyzed proteins from various grasses by Prunty's modification of the Sullivan method (22). The corrected values for the content of cystine ranged from 0.21 (red clover) to 1.20 (lucerne) per cent. The cystine as determined by the Vickery-White method was considerably higher than the cystine as determined by the Sullivan-Prunty method. This suggests the presence of a sulfur-containing amino acid of unknown nature. By enzymatic hydrolysis, cystine was liberated in amounts corresponding to the values obtained by the Sullivan-Prunty method. On the basis of the values obtained, it is calculated that pasturage contains "ample cystine for the wool-protein requirements of the sheep which it normally carries," thus making unnecessary the assumption of cystine synthesis by micro-organisms or by the hair follicle during keratinization, as has been suggested (61).

**Keratins.**—The methionine content of wool (Baernstein method) ranged from 0.44 to 0.67 per cent, or calculated as sulfur from 2.4

<sup>1</sup> Lewis, H. B., *Ann. Rev. Biochem.*, **1**, 177 (1932); **2**, 99, 102 (1933).

to 4.8 per cent of the total sulfur; for camel hair, the corresponding values were 0.66 and 4.7 per cent respectively (62). These findings furnish evidence in support of the belief that in wool and related keratins, as contrasted with the usual type of proteins, essentially all the sulfur is present as cystine.

Although the absence of sulfur from the medulla of hair has been generally accepted,<sup>2</sup> on the basis of the comparison of the composition of medullated and non-medullated fibers or of qualitative microchemical tests, it has not been possible to obtain direct analyses of the two morphological parts of the hair shaft. The discovery of a procedure by which the cortex may be separated from the medulla of goat hair has made direct chemical analyses possible (63). The percentage of sulfur in the cortex thus separated was 3.60 and in the medulla, 0.23. It is believed that the medulla is substantially free from sulfur.

Recent developments have led to investigation of the cystine content of human nails, a type of keratin hitherto little studied (64, 65). Normal nails contained an average cystine content of 10.97 per cent (64) (range 8.77 to 13.38 in 36 individuals) or according to Sullivan & Hess (65), 11.69 per cent (range 10.28 to 13.02 in 26 individuals). In pellagra with extensive dermatitis (14 cases), marked reduction of the cystine content of finger nails (7.26 per cent with a range of 5.03 to 8.55) without appreciable change in total protein content was observed; with subsidence of the dermatitis and clinical improvement, the cystine content returned to normal limits (64). In severe pellagra without dermatitis, normal cystine values were found. This suggests that there occurs in pellagra some abnormality of sulfur metabolism, manifested in the epithelial tissue and related to the dermatitis of pellagra.

Similar low cystine values were observed in arthritis (65). The nails contained 9.77 per cent of cystine (range 7.20 to 13.11 per cent in 103 cases); 65 per cent of the arthritic finger nails were lower in cystine content than the lowest normal value (10.28 per cent). Clinical improvement was accompanied by a return of the cystine content of the nails to normal values. In explanation of these findings it is suggested that there is present, in arthritis, "an intoxication factor which draws on the sulphur complexes as, for example, glutathione, and thus diverts the sulphur from its normal channels which would lead to a finger nail containing at least 11 per cent cystine."

In contrast to the usual type of keratins, the human skin (*stratum*

<sup>2</sup> Lewis, H. B., *Ann. Rev. Biochem.*, 1, 172 (1932).

*corneum*, desquamated epithelium from a case of exfoliative dermatitis) contained relatively small quantities of cystine: 2.31 per cent (Folin-Marenzi) (66).

The disulfide linkages of keratins (wool, chicken feathers) may be reduced by thioglycolic acid in an alkaline medium (pH 10) to form a new protein, soluble in acid or alkali, with a definite optimal point of flocculation and digestible by pepsin or trypsin (67). The only chemical alteration appears to be reduction of the disulfide to sulfhydryl, loss of sulfur or cystine (cysteine) not being observed. The sulfhydryl protein can be reoxidized to the disulfide state and the new disulfide protein is soluble in acid or alkali and digestible by enzymes. Other reducing agents (potassium cyanide, sodium sulfide, sodium sulfite) also form the sulfhydryl protein but secondary changes also occur. The pH is never high enough to dissolve wool of its own accord in a comparable time. To explain the combined effects of alkali and reductant, the following hypothesis is offered:

The cross links between peptide chains in keratin are of two kinds, disulfide links and bridges formed by the electrostatic attraction of the  $\text{NH}_3^+$  group of the diamino acids for the  $\text{COO}^-$  group of the dicarboxylic acids. These salt-like bridges will be broken in alkaline solution by removal of a proton from the amino group. It appears that it is necessary to open these links before the disulfide groups may be reduced.

*Liberation of cystine in protein hydrolysis.*—Colorimetric estimation of cystine (Sullivan) showed a rapid cleavage of cystine from casein in acid hydrolysis (20 per cent hydrochloric acid), 20 per cent of the total being liberated in 30 minutes, 50 per cent in  $3\frac{1}{2}$  hours, and 100 per cent in 6 hours (68). When the cystine was estimated by the less highly specific Folin-Marenzi method, acid hydrolysates of casein showed at first high values followed by a drop in chromogenic values as hydrolysis progressed until, at the end of 18 hours, the value became constant, equivalent to 0.33 per cent of cystine, the cystine content of casein as determined by the Sullivan method. When peptic digests of casein were examined by the Sullivan method, no evidence of cleavage of cystine was obtained, but an early rise and subsequent fall in chromogenic values, as determined by the Folin-Marenzi procedure, occurred similar to those observed in acid hydrolysis. It was suggested that these color values were due either to the reaction of compounds other than cystine or to reactive groups of casein exposed during the initial stages of proteolysis.

In further studies (69), it was possible by simple methods to sepa-



rate the products of a one-hour peptic digest of casein into 3 fractions: fraction A, a flocculent suspension, was removed by centrifugation; fraction B was obtained as a precipitate by adjustment of the pH to 6.0; fraction C comprised the residual material. Fractions A and B, comprising 21.9 and 12.7 per cent respectively of the original material, contained no cystine (Sullivan) and 87 per cent of the total phosphorus. Both fractions, A and B, gave chromogenic values in the Folin-Marenzi cystine method, especially before acid hydrolysis. These studies emphasize the desirability of renewed interest in the investigation of the fractionation of the partial hydrolysis products of protein by the use of the improved methods for the study of proteins. Too little attention has been given to this phase of protein study in recent years and the older observations of Kühne and Chittenden and their pupils have been neglected.

#### METABOLISM OF THE SULFUR-CONTAINING AMINO ACIDS

*Absorption from the alimentary canal.*—The relative rates of absorption from isolated jejunal loops of the dog have been determined for *dl*-cystine, *l*-cystine, cysteine, cysteic acid, and sodium sulfate (70). Cysteic acid was absorbed most rapidly and *l*-cystine and sodium sulfate least rapidly. The rate of absorption of *dl*-cystine was somewhat more rapid than that of *l*-cystine.

As determined by the technique of Cori, the absorption coefficient of *dl*-methionine from the gastro-intestinal canal of the young albino rat (71) was slightly lower (0.359 milli-equivalent per hour per 100 gm. body weight) than the previously determined rates for cystine (0.425 and 0.441 calculated as milli-equivalents of cysteine).

*Oxidation of sulfur in cystine and methionine.*—Of the isomeric forms of cystine, *l*-cystine was readily oxidized to sulfate by the rabbit, while *d*-cystine was oxidized with difficulty (72). Meso- and *dl*-cystine were oxidized to a degree intermediate between that of the *l* and *d* forms. Acetyl- and formyl-*l*-cystine were as readily oxidized as free *l*-cystine, whereas the corresponding derivatives of *d*-cystine were far more resistant to oxidation than *d*-cystine (73). The ease of oxidation of acetyl-*l*-cystine by the rabbit is in contrast to the behavior of acetyl-*l*-cysteine<sup>3</sup> in the dog (74). In this animal, in contrast to *l*-cystine, acetyl-*l*-cysteine was oxidized with difficulty. In consid-

<sup>3</sup> The preparation of acetylcysteine used by Pirie & Hele is designated acetyl-*d*-cysteine in their paper. Since it was prepared from *l*-cystine, it has seemed desirable to designate the product as acetyl-*l*-cysteine.

ering these data, the possibility that the paths of metabolism of cystine and cysteine may not be the same must be considered, particularly in the light of the recent studies of cystinuria to be discussed later.

Studies with adult and growing dogs (75) have shown that the sulfur of *dl*-cystine was not as well retained as that of *l*-cystine and have led to the conclusion that "*d*-cystine sulfur is not used by adult dogs to replace the tissue waste."

The sulfur of *dl*-methionine was readily oxidized by the dog (76) and rabbit (77, 78), while the sulfur of the isomeric S-ethylcysteine (76) was not oxidized to any significant extent. When the amino group of methionine was "blocked" by a benzoyl group, oxidation of the sulfur of the resulting compound did not take place readily (77). A similar failure of oxidation of the sulfur when deamination was prevented by "blocking" the amino group has been observed with cystine. The sulfur of *dl*-homocystine was also readily oxidized (78), as was also the sulfur of S-methylcysteine. After the subcutaneous injection of cystinamine or cysteinamine hydrochloride into dogs, there was a delayed increase in the organic sulfur of the urine which was not due to taurine, cystine, or cystinamine (79). Since the determination of cystine was made according to the method of Folin & Marenzi, not only cystine but -SS- linkages were absent from the urine.

When *dl*-methionine was fed to rats (71) or rabbits (77), evidence was obtained for the presence in the urine of a substance containing the -SS- linkage which was not cystine as the Sullivan reaction in the urine was negative. This has led to the hypothesis that methionine is demethylated and that the product of the demethylation, homocysteine, is excreted after oxidation to homocystine. Further evidence in support of the theory of demethylation as a reaction in the metabolism of methionine is presented in the *in vitro* experiments of Pirie (80), who observed that, when slices of liver or kidney were incubated with methionine, the fluid gave no Sullivan reaction but a positive cyanide-nitroprusside test which was absent in the control experiments. This investigator also has reported the formation of sulfate from methionine and from cysteine by slices of tissue *in vitro*; the formation of sulfate from cystine appeared to require preliminary conversion to cysteine.

Cystine has also been detected in the blood of rats in small amounts; the amounts were increased after feeding (23).

*Mercapturic acid synthesis.*—When  $\alpha$ -naphthalene was fed to rabbits, there was isolated from the urine a compound analogous to

bromophenylmercapturic acid, *l*- $\alpha$ -naphthylmercapturic acid (81). This new mercapturic acid was synthesized and the synthetic product was shown to be identical with the product obtained from urine (82).

Young white rats on a cystine-deficient diet declined in weight when small amounts of bromobenzene were added to the diet (83), a loss in weight which was assumed to be due to utilization of cystine for mercapturic acid synthesis rather than for purposes of growth. Addition of either cystine or methionine to the diet led to resumption of growth.

*Relation of the sulfur-containing amino acids to growth.*—It will be recalled that either cystine or methionine can induce growth in young white rats fed a diet low in its content of cystine. Homocystine, whose formation as a product of the metabolism of methionine has been discussed earlier in this review, also serves effectively as a supplement to a cystine-deficient diet and promotes growth (84). This suggests that the common factor in the growth-promoting action of cystine, methionine, and homocystine may be a  $-SS-$  or  $-SH$  linkage, actual as in homocystine or cystine, or potential as a result of demethylation in methionine. Since S-methylcysteine fails to promote growth (78), it may be assumed that the path of metabolism of this cysteine derivative does not involve demethylation to yield cysteine. The behavior of the recently synthesized homologues of cystine and methionine, pentocystine and homomethionine, in the promotion of growth should be important for the solution of the problem of the specific or non-specific nature of the groups in cystine or methionine, responsible for their growth-promoting activity.<sup>4</sup> In contrast to *d*-cystine, which is not effective as a growth-promoting factor for rats on cystine-deficient diets, *d*- and *l*-methionine are equally effective in stimulating growth (85). Mesocystine is as effective as *dl*-cystine in this respect (86). This is presumably to be explained by reduction of the internally compensated mesocystine by the organism, whereby *l*-cysteine is made available for growth, the *d*-component of the molecule having no growth-promoting properties.

*Cystine and the growth of hair and wool.*—The question of the relation of dietary cystine or sulfur to wool production has been dis-

<sup>4</sup> Studies which have appeared after this paper was written [Dyer, H. M., and du Vigneaud, V., *J. Biol. Chem.*, **108**, 73 (1935)] have indicated that neither pentocystine nor homomethionine can be utilized for growth in lieu of cystine, thus demonstrating that the availability of homocystine and methionine for this purpose is not a general property of either disulfide or methylthiol amino acids.

cussed extensively from the theoretical, experimental, and practical standpoints. Excellent reviews are now available (87, 88, 89). Studies of the relation of dietary methionine to the cystine content of the hair should be of unusual interest in view of the demonstration of the similar functions of cystine and methionine in growth. Study of the therapeutic value of hydrolyzed wool in increasing the growth of hair in rabbits has given indication of positive results and the clinical application of this therapy has been discussed (90).

*Cystinuria*.—Preliminary reports of studies, which may materially alter our conception of the nature of the "inborn error," cystinuria, have been reported (91). As has been observed frequently, the administration of cystine to a cystinuric patient did not alter the excretion of cystine. However, after the administration of cysteine, a marked increase in cystine excretion occurred. When methionine was fed, significant increases in urinary cystine were noted. The administration of homocystine did not affect the cystine excretion, the homocystine sulfur being excreted as inorganic sulfate sulfur. Unpublished experiments in the writer's laboratory have confirmed the results obtained with methionine feeding. While it is idle to speculate extensively in the absence of complete data, these preliminary notes suggest that the reaction  $\text{cystine} \rightleftharpoons \text{cysteine}$  may not be as readily reversible *in vitro* as *in vivo*, that cystine and cysteine do not necessarily follow the same paths in catabolism and that methionine may be metabolized via cysteine. From the standpoint of dietary control of cystinuria, these experiments are suggestive, indicating that the cystine of the urine in cystinuria "may be derived in part or in whole from that portion of the protein sulfur which is present in the protein molecule in the form of methionine."

#### GLUTATHIONE AND SULFHYDRYL COMPOUNDS

Despite the many recent investigations concerned with glutathione, our knowledge of its distribution and function is still limited. Synthesis has not yet been achieved. Cystinyl peptides, of the type obtained by hydrolysis of glutamic acid from the glutathione molecule, have, however, been prepared (92). The lack of a specific and satisfactory method for the quantitative determination of glutathione is a handicap to investigators. The methods most commonly used, modifications of the iodimetric titration method of Tunnicliffe, are not specific, since other reducing substances, notably ergothioneine, cysteine, and most important and widely distributed of all, ascorbic acid

(93, 94, 95), interfere. Thus it has been stated that the glutathione content of tumor tissues as obtained by iodimetric methods may indicate three times as much glutathione as is actually present and that the high iodine titration is due chiefly to the occurrence of ascorbic acid in tumors (95).

A recent method for which a high degree of specificity is claimed (96) involves the precipitation of glutathione by cadmium lactate from trichloroacetic acid extracts of tissues. The cadmium complex is dissolved in phosphoric acid and the glutathione is determined by iodimetric methods. Ascorbic acid and ergothioneine do not interfere and the reaction of cysteine may be avoided by a slightly modified procedure. The values obtained are stated to be much lower than the usual values and to be more uniform than those obtained by the older iodimetric methods. Thus in six samples of dog blood, a range of 11.30 to 13.72 mg. per cent with an average figure of 12.76 is reported. The values found are similar to those obtained by the use of the colorimetric method of Bierich & Rosenbohm (94). The iodimetric method of Okuda has also been modified for the estimation of both reduced and oxidized glutathione in tissue extracts deproteinized with sulfosalicylic acid (97). A reinvestigation of the glutathione content of the lens of the eye has shown that much of the so-called glutathione is ascorbic acid (98).

While the function of glutathione is not clearly understood, the available evidence suggests the rôle of glutathione and sulfhydryl groups as activators or coenzymes for enzyme activity and a loss of activity when the sulfhydryl group is converted to the oxidized ( $-SS-$ ) form. The hypothesis that thiol groups are essential parts of the enzyme itself and that oxidation of these groups to the disulfide form leads to inactivation of the enzyme has also been suggested. It is possible to cite a few only of the many papers concerned with this phase (99 to 105). The inhibitory action of iodoacetic acid on yeast fermentation (106) and upon carbohydrate metabolism of muscle (51, 107) appear to be explained, in part at least, by the combination of the iodoacetic acid with glutathione or some similar compound (51, 52), thereby preventing the coenzyme from functioning.

Thiol compounds (108), including glutathione, cysteine, thiolhistidine, and ergothioneine, increased the coagulation time of the blood. The effect of glutathione was most marked. The peculiar type of clot, observed in progressive obstructive jaundice either clinical or experimental (109), is believed to be due to a disorder of sulfur metabolism,

which leads to an accumulation of sulfur compounds (cysteine?) in the blood. In experimental animals, clinical improvement was obtained on administering bromobenzene on the theory that this substance by combining to form mercapturic acid would remove cysteine or a similar catabolite from the blood.

Evidence that a parallelism exists between the glutathione content of tissues and the inherent capacity of a race of rabbits to attain large, intermediate, or small adult body size has been obtained in breeding experiments (110).

The evidence for and against the theory that the sulfhydryl group is a specific stimulus for growth by increase in cell number<sup>5</sup> has been summarized recently by Hueper (111).

#### SULFUR OF THE URINE

Wilson (112) has again studied the problem of the relation of the excretion of urinary sulfur to nitrogen. In experiments on dogs, three possible explanations of the urinary N : S relationships were considered: (a) the early excretion of sulfur may be due to an excretion of sulfate by the kidneys more readily than urea or ammonia; (b) cystine may be more open to attack than other amino acids; (c) cystine or some unit containing sulfur may occupy a key position in the protein molecule, whereby it is the first split off in catabolism and the first to be retained in anabolism. This last hypothesis is considered most probable.

The various factors which may influence the organic sulfur of urine have been investigated by Amann. The organic sulfur appeared to be fairly constant per unit of body weight for animals of the same species but to vary with the species (113). The effects of variation in the levels of energy and protein metabolism have also been noted (114, 115).

It is maintained that the ethyl sulfide fraction of the organic sulfur of dog urine is endogenous, originating from the catabolism of cystine (116). Evidence in support of the origin of this fraction of urinary organic sulfur from intestinal putrefaction has also been presented (117).

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## THE CHEMISTRY AND METABOLISM OF THE NUCLEIC ACIDS, PURINES, AND PYRIMIDINES\*

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Rapid progress has been made during the last two years in the study of the constitution of the nucleotides and nucleosides obtained from yeast nucleic acid. Levene & Harris (1) have investigated the structure of the ribosephosphoric acid present in yeast adenylic acid. They found it to be identical with that obtained from yeast guanylic acid. It is a *d*-ribose-3-phosphoric acid. According to these workers yeast adenylic acid should be designated 1-adenine-*d*-ribofuranoside-3-phosphoric acid. They suggest for it the name adenosine-3-phosphoric acid. Levene & Stiller (2) have synthesized ribose-5-phosphoric acid and found it to be identical with the product obtained previously by Levene & Jacobs (3) on hydrolysis of muscle inosinic acid. Since inosinic acid may be obtained from muscle adenylic acid by deamination, these findings establish the position of the phosphoryl group in these two compounds. Additional evidence for the identity of the two nucleotides is presented in the paper of Levene, Harris & Stiller (4). Thus, muscle adenylic acid may be designated adenosine-5-phosphoric acid.

A new method of approach to the study of the constitution of the nucleosides is presented in the work of Bredereck (5). He has found that uridine, cytidine, adenosine, and inosine react with tritylchloride, yielding crystalline trityl compounds containing the trityl group in position 5 of the ribose, thus furnishing additional evidence for the assumption of a furanoid structure in these nucleosides. The same method of approach was used in investigating the constitution of the pyrimidine nucleotides (6). Uridylic acid was prepared by deamination of cytidylic acid. The dibrucine salt of uridylic acid yielded with tritylchloride a brucine-trityl-uridylic acid, which, on removal of the brucine, gave a sodium salt of trityl-uridylic acid, in which the trityl group was attached to carbon atom 5 of the ribose. Thus, the phosphoryl group could only be in position 2 or 3. In analogy to the structure of yeast adenylic acid and guanylic acid, it is assumed that in the pyrimidine nucleotides the phosphoric acid is attached to carbon atom 3.

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Direct experimental evidence as to the attachment of the ribose in the pyrimidine nucleosides has been presented by Levene & Tipson (7). They prepared trityluridine and blocked the remaining hydroxyl groups in the sugar by acetylation. On treatment with diazomethane, one methyl group was introduced. The methylated compound yielded on hydrolysis 1-methyluracil, thus showing that the ribose was attached to position 3 of the pyrimidine. In this investigation the assumption was made that tritylchloride reacted with the primary alcoholic group in position 5 of the ribose. Levene & Tipson (8) have subsequently shown that this assumption was correct.

The copper-sulfate test for distinguishing between muscle and yeast adenylic acid, described by Klimek & Parnas (9), has been investigated by Steudel (10). He questions the specificity of the reaction, inasmuch as other nucleotides and also purines and pyrimidines give blue solutions with copper sulfate in the presence of alkali. Further work by Klimek (11) shows that muscle adenylic acid, in contrast to yeast adenylic acid, forms complex compounds with iron, cobalt, and bismuth. As regards the behavior of the purines in this reaction, observations of Parnas & Klimek (12) indicate that the formation of alkali-soluble complex copper salts depends on the presence of a free imino group in the imidazole ring. However, this seems to hold only for certain purines, since 7-methyl xanthine was found to form a soluble compound (13). The action of palladous chloride on purines has been studied by Gulland & Macrae (14). This reagent was found to precipitate purines quantitatively. The authors suggest its use for the separation of purines from each other.

The oxidation of uric acid under various conditions has been studied by several workers. Frèrejacque (15) reports that in the oxidation of uric acid with oxygen in the presence of manganese dioxide and glycine isoallantoinylglycine is formed. When alanine is used, the corresponding alanine compound is obtained. These observations suggest to the author the possibility of a function of amino acids in the oxidation of uric acid *in vivo*. Schuler & Reindel (16) investigated the action of various oxidizing agents on uric acid in an alkaline medium. Only two, namely, potassium permanganate and oxygen in the presence of manganese dioxide, were found to form allantoin, thus simulating the breakdown of uric acid *in vivo*. Oxidation of uric acid with palladous chloride (14) yields parabanic acid, urea, oxalic and mesoxalic acids.

Hammarsten (17) has studied the solubility of alkali urates in

salt solutions of different types and varying concentrations. Some of her findings are as follows: lithium and potassium urates appear to give entirely true solutions at 37°. In saturated solutions of sodium and ammonium urates the urates are partly present in colloidal form. In 0.17 per cent sodium chloride solution (the mean concentration present in urine) the solubility product of sodium urate is increased by about 300 per cent. In the case of ammonium urate a similar increase was found. These observations confirm previous findings (18) to the effect that urates and uric acid in the presence of urates have a marked tendency to remain in supersaturated solution. This tendency, in conjunction with the unusually low rate of crystallization of urates, gives the organism a good protection against an undesirable precipitation of uric acid and urates. Young & Musgrave (19) have investigated the conditions governing the formation of supersaturated solutions of urates, their gelation and crystallization. Two classes of urates were found: one spontaneously forming gels on cooling, the other forming gels only in the presence of electrolytes. A more detailed study of the effect of electrolytes on this phenomenon was carried out by Young, Musgrave & Graham (20).

Richardson (21) has studied the rate of atmospheric oxidation of dialuric acid with reference to pH, temperature, concentration of dialuric acid, and influence of catalysts.

Hilbert (22) has described a method for the preparation of cytosine derivatives alkylated in position 1. He also gives an improved procedure for the synthesis of cytosine and isocytosine based on the method of Hilbert & Johnson (23). Unsuccessful attempts to prepare uracil glycol by various methods are described by Johnson & Dyer (24). They found that it could not be isolated because of its great tendency to pass into isobarbituric acid. Of interest is their observation that isobarbituric acid is formed on reduction of isodialuric acid with the Adams platinum catalyst. The oxidation of uracil *in vitro* has been studied by Schwob & Cerecedo (25). It was found that hydrogen peroxide in the presence of charcoal oxidizes uracil, forming isobarbituric acid, isodialuric acid, urea, and oxalic acid. Under these conditions the oxidation of uracil *in vivo* can be simulated *in vitro*. A new synthesis of thymine is described by Bergmann & Johnson (26). Lieben & Edel (27) have studied the color reaction given by alloxan in the presence of amino acids.

A method for the purification of thymus nucleic acid by means of ultrafiltration is described by Caspersson (28). It is a well-known

fact that allantoin, although containing an asymmetric carbon atom, has been found to be optically inactive, regardless of whether it is prepared by chemical or biological methods. Samples of urinary allantoin were also found to be optically inactive (29). Attempts to effect a resolution of the compound had been unsuccessful. Fosse and his coworkers (30) have now found that allantoinase, from soy beans, converts *d*-allantoin to allantoic acid more rapidly than *l*-allantoin, enabling them to isolate the latter. They also report the isolation of *d*-allantoin from the leaves of *Platanus orientalis* (31). It has also been obtained from calf's urine (32).

#### ANALYTICAL METHODS

Caspersson (33) has determined the optimum conditions for the reaction between thymus nucleic acid and the fuchsine reagent as a basis for a quantitative method.

Thomas & Bulgaru-Puscariu (34) have modified Flatow's method for the determination of uric acid in blood so that it can be used in both urine and blood. Keighley & Borsook (35) describe a modification of the technic of Morris & Macleod for the estimation of uric acid in urine, which permits a rapid analysis of a large number of samples. Pritham & Anderson (36) have compared several blood-uric-acid methods so as to ascertain the most satisfactory for the analysis of uric acid in bovine and avian blood. Rusznyák & Hatz (37) offer a volumetric micromethod for the estimation of uric acid in urine and blood. Fürth & Edel (38) have worked out a technic for the determination of uric acid in tissues. Richards and his coworkers (39) describe an ultramicro adaptation of Folin's method for the estimation of uric acid in glomerular urine. Folin (40) has made a critical study of his colorimetric method for the determination of uric acid in blood and urine.

Schmidt (41) describes a procedure for the estimation of guanine, adenine, and hydroxypurines in one portion of tissue. Kerr & Blish (42) have developed a method for determining nucleotides in blood and muscles. Parnas, Ostern & Mann (43) report a method for the estimation of adenosinetriphosphoric acid.

#### PURINE METABOLISM

Chrometzka (44) has studied various phases of purine metabolism in man. He reports that the administration of uric acid and uric acid precursors produced an increase in the daily total-creatinine output. Following the ingestion of large amounts of yeast nucleic

acid, the allantoin output was found to increase from a daily average of 15 to 20 mg. to 60 to 70 mg. Allantoin is considered to be not an end product but an intermediate in the catabolism of purines in man, since feeding experiments with this substance indicated that it is partly broken down to yield urea. The author claims to have obtained evidence of the presence of two hitherto unknown substances in the urine which seem to play a rôle in purine metabolism. One is a peculiar form of uric acid which is destroyed on hydrolysis. The other seems to be an oxidation product of uric acid, which has not yet been identified. Lastly, previous claims of other workers (45) as to the existence of oxaluric acid in the urine could not be confirmed. However, the administration of nucleic acid was found to cause the excretion of substances which on hydrolysis give rise to oxalic acid. In this connection, it may be mentioned that oxaluric acid has been considered by Cerecedo (46) to be an intermediate link in the catabolism of purines and pyrimidines in the animal body.

Terroine, Champagne & Mourot (47) have compared the amounts of the nitrogenous constituents excreted in the urine by various species under conditions of protein endogenous metabolism. The quantity of allantoin nitrogen excreted by the rat, rabbit, dog, and pig is 13.4, 6.3, 11.9, and 9.8 per cent of the total nitrogen, respectively. Man excretes 4.7 per cent of the total nitrogen in the form of uric acid under the same conditions. The high percentage of allantoin nitrogen in the urine of the dog has already been pointed out by others (48). It is found to be still higher for the rat. In fact, it is higher than the ratio, purine N/total N, of the tissues. The former is 13, the latter 2 per cent. Following these observations, Terroine & Mourot (49) have studied the endogenous purine metabolism of the rat. They found that, during complete inanition as well as protein starvation, the amounts of total purines (allantoin and purine substances) excreted in the urine are much higher than those lost by the organism during the same period. These observations show that under the conditions of endogenous protein metabolism a synthesis of purine substances takes place at the expense of tissue proteins, whereas under normal conditions, as these workers showed previously, part of the purine excreted originates from the protein present in the diet. The low value of the endogenous purine fraction, being only 20 per cent of the total purines excreted, is pointed out.

Degan (50), working in Terroine's laboratory, has found, in experiments on dogs, rabbits, and pigs, that, expressed per unit



of weight, the sum of purine substances excreted in the urine is identical in animals of the same species and of equal size, provided they are kept under conditions of endogenous protein metabolism. On a diet containing protein but free of purines, the total purine excretion varied considerably from animal to animal, but in every case it was found to be higher than on a carbohydrate diet, indicating a synthesis of purine material from protein. These results were substantiated by the further observation (51) that whenever there is a retention of nitrogen in the body, there is a lower output of purines in the urine. The findings of Kapeller-Adler and her co-workers (52) point also to a participation of proteins in the synthesis of purines in the animal body. Degan (53) also attempted to determine whether there is a parallelism between caloric intake and the endogenous purine output. The animals were kept on a protein-free diet, but the amount of food given varied within wide limits. It was found that a four-fold increase in the amount of food ingested did not cause a corresponding rise in the endogenous purine excretion, the level of purine nitrogen eliminated following in general that of the total nitrogen. These results show that the "cost of digestion" does not affect the endogenous purine metabolism to any great extent. That under certain conditions the purine output may not run parallel to that of the total nitrogen is brought out in the findings of Terroine & Champagne (54). They observed that whereas acid substances, when added to the diet, caused an increase in the total nitrogen excretion, they did not affect the creatinine and allantoin output.

The eminent rôle played by the liver in the conversion of uric acid to allantoin is emphasized anew in the experiments of Bollman & Mann (55) on dehepatized dogs. They confirmed their previous findings as regards the fate of uric acid when injected intravenously into such animals. It is almost completely recovered in the urine, in contrast to the small amount recovered under similar conditions in the normal animal. Within nine hours after the injection of uric acid into normal dogs the increase in the amount of allantoin excreted is equivalent to 50 to 90 per cent of the uric acid administered. In the dehepatized dog no such increase was observed. The constantly decreasing elimination of allantoin after removal of the liver is considered as evidence of the cessation of allantoin formation in the absence of the liver. The mechanism of the destruction of uric acid in the normal dog has been investigated by Wilhelmj & Moskowitz (56) by means of blood-uric-acid-tolerance curves following

the intravenous injection of uric acid. The results of single injection experiments showed that when quantities of uric acid ranging from 0.64 to 1.20 gm. were administered to normal dogs very little of the substance was excreted in the urine. The blood uric acid follows a logarithmic curve, the rate of decrease being approximately 3 per cent of the amount present per minute. The results obtained with continuous injections of uric acid showed that the ability of the dog to destroy uric acid is sharply limited. When the rate of inflow of uric acid exceeds the maximal rate at which it can be oxidized, it accumulates in the blood. When a single injection was combined with a continuous injection, the behavior of the blood uric acid was very similar to that observed in man following a single injection of uric acid.

#### URIC ACID SYNTHESIS IN BIRDS

The baffling problem of the mechanism of uric acid synthesis in birds has received considerable attention in recent years. It will be remembered that Minkowski (57), on the basis of his experiments on dehepatized geese, had drawn the conclusion that the liver was essential for the uric acid synthesis. Subsequently, Wiener (58) advanced the hypothesis that urea and a three-carbon compound were the precursors of the uric acid. Schuler & Reindel (59), using tissue slices of the pigeon in their experiments, have found that the process consists of two steps: the first is the formation of a non-purine precursor, caused by an enzymatic reaction taking place in the liver and kidney. This precursor was also found in the muscles and other organs. The second is the synthesis of uric acid from this precursor in the kidney. The source of the nitrogen is the ammonia derived from the amino acids. Urea is not utilized for the synthesis. The source of the carbon is as yet unknown. Glycerol, lactic, malonic, and tartronic acids, considered as precursors by Wiener, are not involved. Similar observations were made for the hen and the goose (60), with the difference that in these species the synthetic process takes place both in the liver and kidney. These findings have been confirmed by Benzinger & Krebs (61). The observations of Russo (62), Clementi (63), and Torrisi (64), who used a different method of approach, have also rendered Wiener's hypothesis untenable.

#### ENZYMES

Studies on the enzymatic activity of the intestinal mucosa of the calf by Klein (65) and of the gastro-intestinal secretions of the dog by Levene & Dillon (66) had shown the presence of two enzymes,

one nonspecific, acting on nucleotides and other phosphoric esters, the other specific, acting on the nucleic acid molecule, a polynucleotidase. Klein (67) has succeeded in inhibiting the action of the nonspecific enzyme by means of sodium arsenate. He proposes for this new polynucleotidase the name "thymonucleinase" since it appears to be specific for thymonucleic acid. This method of separation enabled Klein & Thannhauser (68) to obtain the barium salt of guanine-desoxyribose-phosphoric acid and to study its properties. The adenine nucleotide, in the form of the calcium salt (69), was also obtained. A further fractionation of the enzyme system was carried out by Klein (70). In addition to the thymonucleinase and the phosphatase, previous work had shown a deaminase to be present, which acts on adenine-desoxyribose-nucleoside but not on the guanine nucleoside. Klein has found that silver salts inhibit the action of the deaminase but do not affect the thymonucleinase or the phosphatase. Thus, adenine desoxyriboside could be isolated and its properties studied. On the basis of these findings, the breakdown of thymonucleic acid in the intestine is assumed to take place in the following manner: The first step is the conversion of the polynucleotide into mononucleotides, brought about by the thymonucleinase. This is considered to be a very slow process. In the second step the nucleotides are hydrolyzed to form the nucleosides. This is a rapid process, caused by a nonspecific phosphatase, which prevents the accumulation of any large amounts of nucleotides in the intestine. The nucleosides are assumed to be absorbed as such except the adenine desoxyriboside, which is deaminized prior to its absorption. A similar enzyme system, acting on yeast nucleic acid, is present in the duodenal mucosa of the rabbit, according to the observations of Makino (71).

Further evidence supporting the assumption that hydroxyacetylene-diurein-carboxylic acid is an intermediate in the uricolytic breakdown of uric acid is presented by Schuler & Reindel (72). They found that the action of uricase on uric acid as well as the catalytic oxidation of uric acid by oxygen in the presence of manganese dioxide in alkaline solution yield the same intermediate product, namely, hydroxyacetylene-diurein-carboxylic acid. Kleinmann & Bork (73) have also reported studies on uricase. They found that pig's liver, spleen, pancreas, and bile contain uricase, but were unable to detect the enzyme in human tissues. They confirmed the observations of previous workers that there are two optimum pH's for the hydrolysis, one at pH 8.85, the other at pH 10.0, and that oxygen is essential.

They also observed that the activity of the enzyme is inhibited in the presence of hydrogen sulfide and potassium cyanide, which indicates the presence of heavy metals. In their second paper (74) Kleinmann & Bork reported the purification of uricase by adsorption on kaolin and aluminum hydroxide. Such a purified preparation was found to attack uric acid, giving rise to allantoin, urea, and oxalic acid (75). A simplified method for the preparation of uricase has been described by Truszkowski (76). He finds that lipoids prevent the extraction of the enzyme from fresh tissues. Preliminary studies of the properties of soluble uricase show that hydrolytic activity is indissociable from traces of alkali-soluble proteins. In line with these observations are the unsuccessful attempts of Kleinmann (77) to obtain active uricase preparations after deproteinization of his extracts.

Waldschmidt-Leitz & Köhler (78) have studied the action of kidney phosphatase on glycerophosphate, monophenyl-orthophosphate, hexosediphosphate, and muscle adenylic acid. All these substances were found to be attacked by the enzyme. These observations are similar to those made by Levene & Dillon (79) on the phosphatase of the intestinal mucosa. The unspecific phosphatases of kidney, liver, and bone have also been studied by Jacobsen (80). They are destroyed when heated for a short time at 55° in a weakly acid medium in the presence of ammonium chloride. The specific adenylypyrophosphatase was found to split off only two phosphate groups from adenylypyrophosphoric acid, whereas under the action of the unspecific phosphatases all the phosphoric acid is removed from the molecule. The optimum pH for the action of the specific enzyme was found to be 7.2. It is present in the spleen, lung, pancreas, skeletal muscle and cardiac muscle.

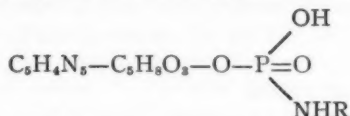
#### COZYMASE<sup>1</sup>

Further work on cozymase has been reported by Myrbäck (81). Kidney phosphatase was found to inactivate cozymase, the inactivation going parallel with the liberation of phosphoric acid from the molecule. It is six times more active than bone phosphatase in this respect (82).

On the assumption that during the inactivation of cozymase by heat a linkage between the amino group of adenine and some other group in the molecule might be attacked, comparative studies on the deamination of active and inactivated cozymase were carried out. The speed of deamination was found to be the same in both cases. Molecu-

<sup>1</sup> Cf. also this volume, pp. 31, 48. (EDITOR.)

lar weight determinations of cozymase (83) gave figures between 450 and 500, showing it to be higher than that required for adenylic acid. Although the substance gives the copper reaction (84), characteristic for muscle adenylic acid, the molecular weight determinations and the ratio of purine nitrogen to total nitrogen content of cozymase preparations show that there is a group containing one atom of nitrogen attached to the adenylic acid. This is also borne out by the results of the hydrolysis of cozymase as compared with those obtained on hydrolysis of adenosine and adenylic acid. The former yields a certain amount of ammonia, while in the case of the latter no detectable amounts of ammonia are formed. A further support for this structure is given by a comparison of the titration curves of active cozymase and cozymase rendered inactive by boiling (85). It was found that the inactive preparation required more alkali for its neutralization than the active one. The inactivation may be also effected by heating with very weak alkali for a short period. The resulting inactive cozymase reacts like a dibasic acid in contrast to the active substance which requires only one equivalent of alkali for neutralization. This dibasic acid seems to be identical with adenylic acid. Inasmuch as the active cozymase reacts as a monobasic acid, the assumption is made that one of the hydroxyl groups of the phosphoric acid is involved in the linkage of the nitrogen-containing group attached to the molecule. On the basis of these findings, Myrbäck proposes the following structure for cozymase:



A substance very similar to cozymase acts as a coenzyme in the dehydrogenation of lactic acid and  $\beta$ -hydroxybutyric acid by heart-muscle tissue (86). It is not identical with muscle adenylic acid or adenylypyrophosphoric acid. An activating effect of cozymase on the enzymatic dehydrogenation of malic, lactic, citric, and glutamic acids and on ethyl alcohol has been observed by Andersson (87).

#### ADENYLPYROPHOSPHORIC ACID (ADENOSINETRIPHOSPHORIC ACID)

The problem of the structure of adenylypyrophosphoric acid still awaits solution. It was pointed out in the previous review (88) that Lohmann's objections to Barrenscheen's formula were based mainly

on two facts: first, the equal speed of deamination of muscle adenylic acid and adenosinetriphosphoric acid with nitrite, and second, the isolation of inosinepyrophosphoric acid. Barronscheen and his co-workers (89) have confirmed their previous observations regarding the deamination of the pyronucleotide. They doubt the existence of inosinepyrophosphoric acid, postulated by Lohmann, inasmuch as they were unable to prepare it, using Lohmann's own method. However, inosinepyrophosphoric acid has been obtained by Mozolowski & Sobczuk (90), by Ostern & Mann (91), and by Kiessling (92), so that there seems to be no doubt as to its existence. A third objection of Lohmann against Barronscheen's formula was based on the fact that, according to his observations, conditions could be found under which the removal of ammonia from the compound by enzymatic action preceded that of phosphoric acid. Mozolowski & Sobczuk (90) report that they were unable to obtain such conditions when working with muscle pulp obtained from frog muscles that had been frozen in liquid air. Jacobsen (93) has investigated the hydrolysis of adenylypyrophosphoric acid in inactivated muscle extracts under various conditions. In a slightly alkaline reaction (pH 9.0) more ammonia than pyrophosphate is liberated from the pyronucleotide, while from neutral or slightly acid extracts the two groups are liberated in the ratio 1:1. The deamination of adenylic acid is twenty times as fast as that of adenylypyrophosphoric acid.

#### HEART NUCLEOTIDE

The existence of an adenylypyrophosphoric acid in the heart, different from that found in skeletal muscle, has been pointed out by Lohmann (94) and by Embden (95). Ostern (96) reports a method for the preparation of the nucleotide from horse's heart. His observations show that the ratio of total nitrogen to hydrolyzable ammonia nitrogen in the compound is 5:1, corresponding to the ratio found in adenine. The substance is quite easily deaminized by muscle extracts. This points to its nucleotide structure. The ratio of total phosphorus to total nitrogen was found to be 5:10. In the light of these findings, the assumption is made that the heart nucleotide is a di-adenosinetriphosphoric acid, consisting of one molecule of adenosinediphosphoric acid and one of adenosinetriphosphoric acid.

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## CARBOHYDRATE METABOLISM\*

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The present review deals, with few exceptions, with articles published in 1934. Earlier papers are not quoted in the bibliography and are given with the year of publication in parenthesis. In order to avoid duplication, several topics discussed in the review article of 1933, notably glycolysis, have been left out. The limited space permitted discussion of only 40 per cent of the papers published. The question of the influence of the pituitary and adrenals on carbohydrate metabolism continued to hold the center of attention. Resynthesis of muscle glycogen in the diabetic animal has been investigated very thoroughly and the mechanism of absorption of glucose from the intestine has received further clarification. It should also be recorded that in the past few years no real advance has been made in regard to the mechanism of insulin action on carbohydrate metabolism. Summaries on various phases of carbohydrate metabolism have appeared by Best, Lucke, and Macleod.

### GLYCOGEN

Dambrosi found that extirpation of the thyroid, parathyroids, gonads, or pituitary did not retard resynthesis of glycogen after tetanic stimulation of muscle. After pancreatectomy a delayed resynthesis was noted. Injection of insulin or transplantation of a pancreas into the neck, before stimulation of muscle, led to good resynthesis. Vagotomy or sympathectomy or both did not retard resynthesis of glycogen in previously fatigued muscle. Foglia & Fernandez injected glucose after double vagotomy and observed a rise in muscle glycogen. A central vagal control of insulin secretion was not found to be essential for glycogen formation in muscle.

Lukens determined resynthesis of muscle glycogen in depancreatized cats from which insulin had been withheld for forty-eight hours. For one to two hours after stimulation, during which time lactic acid and hexosemonophosphate are available for resynthesis, there was no difference between depancreatized and normal animals.

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In later stages of recovery, during which muscle glycogen is formed chiefly from blood sugar, a delay in resynthesis was noted in the muscles of the diabetic animals, but twenty-four hours after stimulation they had regained as much muscle glycogen as the control animals. These findings show that insulin merely serves to accelerate glycogen formation in muscle but is not essential for its formation. They also explain why a diabetic animal is able to maintain good glycogen stores in its muscles in spite of muscular activity.

Butsch administered glucose to unanesthetized dogs by means of continuous intravenous injection. After long periods of injection, rather abrupt rises in blood and urine sugar were noted and these coincided with maximal glycogen storage in the tissues. Glycogen values in the liver of 18 to 22 per cent, and in muscle of 2 to 4 per cent, were observed and these could not be increased further by continuing the glucose injection. In cases of abnormal glycogen storage (Gierke's disease) Biedermann & Hertz observed an almost normal blood-sugar curve after administration of glucose and fructose, and it may therefore be assumed that in this disease the glycogen stores of the body are not filled to capacity.

Olmsted & Read noted that the increase in liver glycogen which occurs in decapitated fasted cats is accompanied by a higher glucose concentration in the hepatic than in the portal blood; hence, the liver glycogen cannot be formed from blood sugar. (It has been suggested that under these conditions the liver glycogen might be formed from blood lactic acid derived from muscle glycogen.) Mackenzie found that rats in which 30 to 50 per cent of the liver had been extirpated, deposited, after a glucose meal, about the same amounts of glycogen in the liver as normal controls.

Sweeney *et al.* noted a low glucose tolerance in rabbits treated with diphtheria toxin. They showed that this was due to a decreased effectiveness of the animals' own insulin, since doses of injected insulin, which in normal rabbits markedly lowered the blood sugar, failed to do so in these toxemic rabbits. Corkill & Ochoa found that such toxemic rabbits, while still capable of forming liver glycogen from injected glucose, are no longer able to form it from injected lactate. Buell & Strauss found that experimental hyperthyroidism in rats impaired glycogen formation in the liver from lactate.

Deuel *et al.* found that male and female rats containing equal amounts of liver glycogen at the beginning of a fast, differ during fasting in that the male animals retain their glycogen longer than

the female animals. In guinea pigs, however, the opposite condition obtained. Greisheimer & Goldsworthy noted that the average blood sugar after fasting periods of twelve, thirty-six, and sixty hours was lower in women than in men.

Somogyi described a simple method for the preparation of phosphorus- and nitrogen-free glycogen. Bell & Young reduced the ash content of glycogen by precipitation with 80 per cent acetic acid and found no difference in the chemical behavior of glycogen prepared from livers of fasted or fed rabbits and rats. Treatment with strong alkali produced no detectable chemical change in glycogen. Tsai, and Carruthers & Ling found that repeated extraction of the liver with trichloroacetic acid and water still leaves a small quantity of glycogen (about 0.2 per cent) which cannot be extracted. The reviewers observed in 1932 that only 33 to 46 per cent of the glycogen present in the rat gastrocnemius can be extracted by exhaustive treatment with trichloroacetic acid (unpublished experiments). Willstätter & Rohdewald refer to the unextractable glycogen as "desmoglycogen" and assume that it is firmly bound to protein.

#### ADRENALS

After double adrenalectomy, resynthesis of glycogen in previously fatigued muscle was retarded and injection of glucose did not materially improve resynthesis. Treatment of the adrenalectomized animals with cortical extract led to normal or even supranormal resynthesis. The same extract did not improve resynthesis when injected into normal dogs (Dambrosi *et al.*) and did not cause deposition of muscle glycogen in depancreatized dogs (Fernandez *et al.*). Insulin, however, led to deposition of muscle glycogen in adrenalectomized dogs. The lactic acid metabolism of stimulated muscle following adrenalectomy has been studied by Nachmansohn, and Cope *et al.*

G. T. Evans (1) found that fasted rats maintained at low atmospheric pressure show an increase in liver and muscle glycogen, while adrenalectomized rats maintained under the same conditions do not show this rise. The cortex is apparently involved in this new formation of glycogen because rats with denervated adrenal medulla behaved like normal animals. (For a discussion of the relation of the adrenal cortex to carbohydrate metabolism, see Britton & Silvette.)

Banerji & Reid found that the glucose tolerance of rabbits with long survival after adrenalectomy was the same as that of normal

rabbits. The slightly lowered fasting blood sugar they ascribe to lack of epinephrine secretion. That epinephrine is secreted during fasting is suggested by the experiments of Dragstedt, who diverted the blood of the adrenal veins into the portal vein and observed an increase in fasting blood sugar. Bodo *et al.* obtained evidence for epinephrine secretion in dogs injected with morphine and undergoing a laparotomy, as shown by changes in liver and muscle glycogen, blood sugar and lactic acid.

Goldblatt (1929) discovered that injection of insulin in young rabbits led to an increase in liver glycogen and attempted to show that this was not due to a secondary output of epinephrine as suggested by others. Cope & Corkill now find that the increase in liver glycogen does not take place when insulin is injected into adrenalectomized young rabbits kept alive with cortical extract. The ability of such animals to form liver glycogen is not impaired, as shown by the effects of injection of glucose, lactate, or epinephrine, each of which caused an increase in liver glycogen. It may be concluded, therefore, that the phenomenon observed by Goldblatt after insulin injection is of adrenal origin. Still another effect which was originally ascribed to insulin has been shown to be due to a reflex discharge of epinephrine elicited by the hypoglycemia, namely the lowering of blood amino acids. Davis & Van Winkle found that this lowering no longer occurs when insulin is injected into adrenalectomized rabbits.

Evidence for increased epinephrine secretion during insulin hypoglycemia in man in cases of Raynaud's disease has been presented by Freeman *et al.* and by Heinbecker & Bishop. Brooks found that spinal cats with denervated adrenals are hypersensitive to insulin and he assumes that there exists a spinal mechanism which activates the adrenal system.

#### PITUITARY

Several authors reported that injections of anterior pituitary extracts led to changes in carbohydrate metabolism.<sup>1</sup> Crude extracts were used which probably contained all the hormones of the anterior lobe, as well as partially purified preparations. Extracts which were enriched in growth or in thyrotropic hormone were effective, but so far a fraction has not been isolated which influences carbohydrate metabolism alone. Apart from the confusion created by the

<sup>1</sup> Cf. also this volume, pp. 297, *et seq.* (EDITOR.)

differences in the extracts used, some workers described acute effects on carbohydrate metabolism, while others noted changes only after prolonged injections. Where acute effects were observed the possibility that epinephrine secretion might be involved has not always received sufficient attention. In the case of prolonged injections of crude extracts rather large amounts of protein were administered to the animals.

In four dogs injected with preparations of growth hormone by H. M. Evans *et al.* for long periods of time, two (one male and one female) developed hyperglycemia and glycosuria. In both cases skin infections were present. Houssay *et al.*, who observed that glycosuria developed in dogs after several days' treatment with large quantities (7 cc. per kilo intraperitoneally) of a crude anterior-lobe extract, still obtained this effect after castration, denervation of the adrenal medulla, and to a lesser extent after thyroidectomy. Failure of a dog to respond with hyperglycemia to an active extract has not been reported from Houssay's laboratory. On the other hand, hyperglycemia was never observed on the first or second day of the injections; the average blood-sugar content after the injections had been continued for five to seven days, was 177 mg. per cent (variations from 122 to 300). When glycosuria was present it varied from 0.75 to 2.75 gm. of glucose per kg. per day. In two experiments cats were found to react very strongly to the injections. Guinea pigs, rabbits, rats, and mice were found to be quite unreactive. In rats and mice the hyperglycemia was moderate even though 50 cc. per kg. were injected. Barnes & Regan produced glycosuria in seven out of eleven dogs injected with anterior-lobe extracts, while six thyroidectomized dogs did not respond with glycosuria. Biasotti found a blood-sugar curve of the diabetic type in glucose-tolerance tests on dogs following injection with anterior-lobe extract for six to seven days; there was no rise of the respiratory quotient after the glucose injection. The same results were obtained in thyroidectomized dogs receiving anterior-lobe extracts. In view of these findings, it seems paradoxical that a diabetic glucose-tolerance curve was also obtained in hypophysectomized dogs. Dogs with a lesion of the tuber cinereum of the brain also showed a decreased glucose tolerance.

Eitel *et al.* observed a decrease in liver glycogen of rats two hours after injection of a thyreotropic hormone preparation; this effect was absent in thyroidectomized animals. Loeser gave continued injections of thyreotropic hormone to guinea pigs and found a decline in liver



glycogen in four days, which reached its maximum in seven to eleven days. Holden found the lowest values for liver glycogen in guinea pigs after injections for six days, followed by a recovery to slightly subnormal values in spite of continued administration of the extract. Hyperglycemia, reaching its maximum eight hours after injection, was also noted. According to Silberstein & Gottdenker, the blood sugar rises in normal as well as thyroidectomized cats two hours after injection of a thyreotropic hormone, and on continuing the injections the blood-sugar response becomes less. Jonaš found no change in blood sugar in man after injection of thyreotropic hormone, but glucose ingestion led to a diabetic type of blood-sugar curve and to glycosuria. Fluch *et al.* perfused the livers of frogs several weeks after hypophysectomy and observed a diminished glycogenolysis. Cope & Marks found that the hyperglycemic response to injected epinephrine is diminished in hypophysectomized rabbits. They attribute the hypersensitivity of such animals to insulin to a failure of the secreted epinephrine to restore the lowered blood sugar to normal. Injections of anterior-lobe extracts produced converse changes, namely, an increased resistance to insulin and an increased response to epinephrine.

Spontaneous hypoglycemia following hypophysectomy has been described by several authors in different species. It seems to occur very rapidly in young dogs (Mahoney). Marenzi found normal blood-lactic-acid values in hypophysectomized dogs and a rise in blood lactic acid after injections of anterior lobe extract into normal dogs.

#### PANCREATIC DIABETES

Himwich *et al.* (1) determined the respiratory quotient of muscle, *in situ*, forty-eight to seventy-two hours after pancreatectomy. In sixteen observations on eleven dogs the quotients varied between 0.5 and 1.24. The quotients below 0.65 (three) may have been due to ketone-body formation in muscle. In twelve experiments with quotients over 0.7 the muscles retained ketone bodies in ten instances, suggesting the possibility that the higher quotients were due to oxidation of ketone bodies. Hédon describes, in elaboration of a previous report, a premortal rise in the respiratory quotient of depancreatized dogs (maximal rise to 0.86) associated with a drop in blood and urinary sugar. This rise is observed only after a survival period of at least three weeks, during which time a loss of about 50 per cent of the body weight takes place and the dogs become very weak. Dogs

with acidosis or infections survived too short a time to show this phenomenon. In the absence of other possible explanations the high quotients are explained by carbohydrate oxidation. Clinical cases of severe diabetes, treated by fasting in the pre-insulin era, have shown striking similarity to the observations recorded by Hédon. The experiments discussed below suggest an explanation for the amelioration of diabetes in its terminal stage.

Barnes *et al.* (1) observed an amelioration of pancreatic diabetes in dogs and a decreased insulin requirement after unilateral adrenalectomy, whereas Lewis & Turcatti, and Leloir (1) saw no improvement of the diabetes after extirpation of one adrenal and destruction of the medulla of the other. Hartman & Brownell noted an improvement of diabetes in cats after removal of the second adrenal. Injections of cortin increased the hyperglycemia and glycosuria in depancreatized and adrenalectomized animals. Long & Lukens (1) found that double adrenalectomy had a marked alleviating effect on pancreatic diabetes in cats. In a depancreatized dog which survived five weeks, extirpation of one adrenal was ineffective, while complete adrenalectomy lowered the insulin requirement to one-fifth its previous value. Cortical extract was administered to this dog as well as to the cats in order to prevent deficiency symptoms.

In confirmation of Houssay, Képinow found normal blood sugar or mild hyperglycemia in pancreatectomized hypophysectomized dogs. Injections of anterior-lobe extract caused marked hyperglycemia in these animals. Long & Lukens (2) maintained two hypophysectomized depancreatized cats alive for thirty-five and eighty-five days respectively without injecting insulin. The animals lost 50 per cent of their body weight and excreted some sugar in the urine, but did not develop ketonuria and showed a fairly good food intake. Animals which were merely depancreatized died in from two to seven days after withdrawal of insulin.

Since it has now been shown that the adrenals (presumably the cortex), and also the thyroid, participate in the production of diabetic symptoms, the question arises whether the atrophy of these organs following removal of the pituitary is, perhaps, a sufficient explanation for the amelioration of diabetes. The idea that the anterior lobe elaborates a separate diabetogenic hormone rests on the production of glycosuria in normal animals by injection of extracts, but as indicated before it is not clear whether these extracts act directly or by way of other organs of internal secretion. According to Barnes

*et al.* (2) and Nelson & Overholser, amniotin and oestrin improve pancreatic diabetes by suppressing the diabetogenic activity of the anterior lobe.

#### HEART

Cruickshank & Startup report that the primary defect of the diabetic heart is its failure to oxidize carbohydrate. The isolated hearts of diabetic dogs show respiratory quotients of 0.7 which are raised to unity, after addition of insulin, without change in oxygen consumption (equicaloric replacement of fat oxidation by carbohydrate oxidation). C. L. Evans (1933) reported that the heart in a heart-lung preparation absorbs lactic acid which is supplied by the glycolytic activity of the erythrocytes. C. L. Evans *et al.* now report that the lung also adds lactic acid to the blood in such a preparation.

G. T. Evans (2) determined the glycogen content of the rat heart under a variety of conditions. The most potent factor leading to a decrease in glycogen is asphyxia. Hence, the greatest care is necessary in sampling the heart for glycogen analysis. Whereas epinephrine injections lower muscle glycogen without decreasing heart glycogen, periods of oxygen deficiency (by allowing the animals to breathe 5 to 7 per cent oxygen) decrease the heart glycogen very markedly without affecting muscle glycogen. Recovery of glycogen in a partially asphyxiated heart is very rapid. Himwich *et al.* (2) found that coronary occlusion causes the infarcted area to lose glycogen, which appears as lactic acid and sugar in the circulation. Before coronary occlusion the heart removes lactic acid from the circulating blood. According to Rühl a decrease of about 30 per cent in the oxygen consumption of the heart (brought about by various means) does not decrease the lactic acid absorption of the heart and often increases it owing to increased coronary circulation. Only during almost complete anoxemia does the heart give off lactic acid. The glucose uptake of the heart is slight at normal blood-sugar levels, but becomes marked during hyperglycemia.

Gottdenker & Wachstein found an increased survival time in nitrogen-Ringer solution for strips of rabbit auricle when glucose was added, and Gaddie & Stewart found that an anaërobic frog ventricle, stimulated electrically to exhaustion, is revived by addition of glucose and mannose. All other sugars and certain amino acids were ineffective. Pyruvic acid and glycerophosphate combined, produced slight recovery, while either alone produced no effect.

## ABSORPTION FROM THE INTESTINE

Magers, by comparing the effect of glucose given orally and by means of a duodenal tube, concluded that the rate of emptying of the stomach, ordinarily, has little effect on glucose-tolerance tests in man. Gellhorn & Moldavsky perfused the blood vessels of frog intestine with Ringer's solution of varying pH and found that a change in reaction, either to the acid or alkaline side, reversibly increased the absorption of glucose from the gut. Abderhalden & Effkemann found that various  $\alpha$ - and  $\beta$ -glucosides had a phlorhizin-like effect, i.e., they inhibited phosphorylation in a frog-muscle extract, re-absorption of glucose in the frog kidney, and absorption of glucose in the rat intestine.

In view of the linear absorption rate, observed in experiments on rats in which the gastro-intestinal tract functioned as a unit, it was concluded that the rate of absorption is, within wide limits, independent of the concentration and absolute amount of glucose present in the intestine [Cori (1925)]. This conclusion has now been fully substantiated by experiments of Trimble & Maddock on dogs. They injected glucose solutions directly into the duodenum of unanesthetized dogs and varied the concentrations from 3 to 32 per cent and the absolute amounts from 11 to 65 gm. Varying these factors did not change the rate of absorption to a significant extent and there was a linear relation between absorption rate and time. The average amounts absorbed in grams per kilo per hour for absorption periods of one-half, one, and two hours were 0.91, 0.90, and 0.92, respectively. MacKay *et al.*, on the other hand, again find that more glucose is absorbed in the first hour than in subsequent hours and they relate this to decreasing amounts of glucose entering the intestine from the stomach. The experiments were performed on rabbits to which 30 cc. of 5 per cent agar was fed, so that the stomach and intestine contained considerable amounts of swollen agar when analyzed for their sugar content. According to MacKay *et al.* an average of 1.55 gm. of glucose per kg. per hour was absorbed during the first hour, while the writers, in three experiments performed in 1925 on rabbits fasted for three days, obtained an average absorption of 0.85 gm. during the first hour, a figure similar to those obtained by MacKay *et al.* for the three- to six-hour absorption periods.

## VARIOUS SUGARS

Harding *et al.* (1) fed fructose to normal men and observed low fructose values in the blood (less than 5 mg. per cent) and a definite rise in blood glucose. After ingestion of 25 to 50 gm. of mannose this sugar could not be detected in the blood and there was no rise of blood glucose. One hour after feeding galactose to rats, Harding *et al.* (2) found an average of 33 mg. per cent of galactose in muscle and of 114 mg. per cent in liver. Rats kept previously on a diet rich in galactose and then fed acetoacetate excreted a smaller amount of ketone bodies in the urine than rats kept on a diet rich in glucose (Butts).

Wierzechowski *et al.* found the same specific dynamic action for glucose and fructose when these sugars were given to dogs by means of continuous intravenous injection. Galactose had very little specific dynamic effect. Amytal did not depress the assimilation of intravenously injected sugars. However, a laparotomy and bleeding under amytal anesthesia lowered the tolerance for glucose and maltose, while the assimilation of fructose and galactose was not affected. Galactose infusion did not raise the blood lactic acid but glucose and maltose caused a moderate, and fructose a marked rise. During fasting, as well as during infusion of glucose, galactose, and maltose, the muscles added lactic acid to the blood and the liver retained it. During fructose infusion the liver also added lactic acid to the blood.

Carr & Krantz found that feeding of inulin and of dulcitol leads to the deposition of glycogen in the liver of the rat. In a case of stones in the pancreatic duct, Baranowski & Mozolowski isolated saccharose from the urine.

Abe found that partially diabetic dogs utilize fructose better than glucose, and, according to Kosterlitz, diabetic dogs form more liver glycogen from fructose than from glucose. Bollman & Mann also observed a partial utilization of fructose in depancreatized dogs but when the feeding was continued for ten to fifteen days all the fructose appeared as glucose in the urine. Roe *et al.* found a rise of the respiratory quotient in normal, but not in depancreatized dogs, after feeding 5 gm. of galactose per kg. Drury & Salter found numerous glucose derivatives to be ineffective in the maintenance of life of hepatectomized rabbits.

## VARIOUS DRUGS

Leloir (2) found that the usual hyperglycemic effect of nicotine is absent in adrenalectomized dogs, while it is still present in dogs with denervated adrenals, showing that nicotine acts directly on the adrenal medulla. Dill *et al.* observed no change in blood sugar and blood lactic acid after tobacco smoking, while Haggard & Greenberg confirmed previous reports of a slight rise of blood sugar. Ro investigated the effect of seven opium alkaloids on the blood sugar of rabbits. He found that the phenanthrene derivatives produced a stronger hyperglycemia than the isoquinoline derivatives. After continued administration, the blood-sugar response disappeared; when the injections were discontinued a rise in blood sugar was noted which is regarded as an objective abstinence symptom. Cutting the splanchnics decreased the blood-sugar response (except in the case of codein), and adrenalectomy abolished it.

## INSULIN AND EPINEPHRINE

Ranquist concluded that changes in blood sugar and glucose tolerance, occurring after three to five days and disappearing again within two to three weeks after section of the vagi, are not due to elimination of insulin-secreting fibers, because similar changes are observed after sham operations without section of the vagi. Himsworth, experimenting on rabbits, assumes that the low carbohydrate tolerance during fasting or on a high-fat diet is due to a diminished susceptibility of the animal to insulin rather than to an insufficiency of insulin secretion. The theory is advanced that the insulin secreted by the pancreas must be activated by an unknown tissue factor. Issekutz & Szende arrive at a similar conclusion in their experiments on the perfused frog liver. They had noted that when insulin is injected into a frog fourteen hours previously, the liver of such an animal gives off less sugar upon perfusion than that of a normal frog. The time necessary to bring out this insulin effect could be materially shortened by keeping the frogs at a temperature of 30 to 33° after the injection; with this shortened experimental period it became possible to see whether perfusion of the isolated liver of a normal frog with insulin-Ringer's solution at 30° would result in an inhibition of glycogenolysis. This was found not to be the case and hence it is assumed that the participation of other tissues is essential.

A new explanation for the blood-sugar curve after administration of carbohydrate has been offered by Soskin *et al.* (1). They conclude that the presence of the liver is essential for the normal glucose-tolerance curve, while the pancreas is not essential. Hepatectomized dogs receiving a constant injection of glucose sufficient to maintain a normal blood-sugar level were given an intravenous injection of glucose and showed a "diabetic" type of blood-sugar curve, while depancreatized dogs receiving a constant injection of glucose and insulin showed a normal blood-sugar curve after the intravenous injection of glucose. According to these authors, the liver, in response to the hyperglycemia, decreases the supply of blood sugar, an adjustment for which insulin, but not necessarily an extra supply of insulin from the pancreas, is required.<sup>2</sup> Since insulin accelerates the disappearance of blood sugar in hepatectomized animals and increases glycogen deposition in muscle, the liver mechanism cannot be the only factor determining the blood-sugar curve after administration of glucose. The experiments on depancreatized dogs might be explained by the fact that the amount of glucose metabolized, per unit of insulin, does not remain constant but increases with an increased supply of glucose. This possibility is, however, dismissed by Soskin *et al.* They state that normal glucose-tolerance curves were obtained in hypophysectomized depancreatized dogs receiving no insulin for weeks. On the other hand, Biasotti (2), in Houssay's laboratory, reports that such dogs show a diabetic type of blood-sugar curve upon intravenous injection of glucose.

Hrubetz made a statistical study of the blood sugar of rats after various doses of insulin. The percentage drop in blood sugar was proportional to the dose up to one-half unit per kilo. Since the advent of "true" blood-sugar methods it has become obvious that older values given in the literature for the convulsive level of blood sugar are incorrect. Hiller, Linder & Van Slyke (1925) showed, by means of yeast fermentation, that the true blood sugar is practically zero when rabbits have insulin convulsions and this has now been confirmed in a large series of experiments by Dotti.

Barnes *et al.* (3) found that dogs, made hypersensitive to insulin by denervation of the adrenals or by hypophysectomy, can be protected against two to four times the convulsive dose of insulin by a

<sup>2</sup> The fact that insulin inhibits glycogenolysis in the liver has repeatedly been emphasized by others.



simultaneous intravenous injection of epinephrine at a constant rate. According to Soskin *et al.* (2) a constant intravenous injection of epinephrine increases rather than decreases the uptake of sugar in muscle. They observed in their experiments very marked changes in blood flow through muscle and in the water content of blood during its passage through muscle, which they attribute to the action of epinephrine. There is evidence that in their experiments most of the epinephrine was destroyed before it reached the animal. The writers (in unpublished experiments) secured a marked hyperglycemia in dogs with one-eighth the rate of injection of epinephrine used by Soskin *et al.* Such a rate of injection does not increase blood pressure and there occurs no significant change in blood flow or hydration of blood during its passage through muscle. Strandell found that exercise flattens the blood-sugar curve after glucose ingestion. Exercise had no effect, however, on the blood-sugar curve after epinephrine injection.

Hegnauer & Cori found that immersion of frog muscle for two to three hours in Ringer's solution, containing epinephrine in a concentration of 1 to  $10^6$  to 1 to  $10^8$ , causes a rise in hexosephosphate and lactic acid, which is greater under anaërobic than aerobic conditions. This rise is not inhibited by ergotoxine, or by previous treatment of the frogs with insulin. On transferring the muscles to Ringer's solution without epinephrine, the hexosephosphate disappeared at a much greater rate under aerobic than anaërobic conditions (Cori & Cori). That ergotoxine does not prevent the effect of epinephrine on muscle glycogen was also shown in experiments of Corkill *et al.* on cats. These authors showed further that stimulation of the sympathetics leads to a decrease of muscle glycogen. This effect, however, was not obtained in all experiments (five positive, three negative). Vasomotor responses were always obtained. In two experiments in which ergotoxine was injected, vasoconstriction and decrease of muscle glycogen did not occur after sympathetic stimulation.

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## FAT METABOLISM\*

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In accordance with the editorial policy of the *Review* the present survey is not only an attempt to critically appraise the recent literature on fat metabolism, but to outline the present status of the knowledge and theories of the more outstanding questions in this field. Adherence to this policy has perhaps given to many of the discussions which follow, a rather personal and arbitrary character. Owing to the limited space available only a few special topics have been considered, and even within the limits artificially assigned to these topics, some valuable works are only superficially mentioned, while many others are omitted. In fact, less than one-half of the papers read and abstracted can be quoted.

The following subjects are totally excluded from this review: qualitative and quantitative analysis of lipids in animal tissues, plants, molds, and bacteria; blood and tissue esterases; action of vitamins, drugs, and hormones (including the so-called "fat-metabolism hormones"); pregnancy and lactation; anemias; lipoidosis; fat-deficiency and other diseases; lipids and immunity; cancerous tissues. Some of these topics will be better considered by other reviewers, while others, it is hoped, will be treated in later volumes. Though the present review is essentially based upon papers which appeared between the end of 1933 and October, 1934, some earlier works, which are more strictly concerned with the subjects chosen for discussion, will also be reported.<sup>1</sup>

### FAT DIGESTION

Nothing essentially new has lately appeared to modify the most generally accepted views on this topic. The digestibility of hydrogenated oils under the action of pancreatic lipase was found by Tofte to be inversely proportional to their melting-points, the differences

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<sup>1</sup> Older papers, already mentioned by the former writer of this review, are quoted with the year of publication in parenthesis, but are not given in the bibliography. Preliminary communications are reported only when complete works have not yet appeared.

being reduced by increased temperature; the degree of unsaturation is without effect. The latter conclusion is in agreement with the experiments of Peretti & Tore upon the pancreatic digestion of natural or artificially modified fats with iodine numbers varying between 10 and 180.

Confirming previous investigations, Reale found that the secretion juice of a dog, bearing a Thiry-Vella fistula, is quite active upon the triglycerides of both the higher and lower fatty acids. The mean value of the lipase: esterase ratio (as defined by Willstätter and associates) is about  $Q = 0.3$ , thus conferring upon the intestinal esterase an intermediate place between a true lipase (for that of the pancreas,  $Q = 1$ ) and a true esterase (for that of the liver,  $Q = 0.01$ ). The enzyme is mainly present in the formed elements suspended in the juice, but it easily diffuses into the liquid phase, where it forms a colloidal solution.<sup>2</sup>

#### FAT ABSORPTION

Earlier knowledge of this phenomenon, together with the disturbances caused by disease, are discussed by Wendt, while in two more recent papers by Verzár the physiological factors, upon which the process is dependent, are analyzed. According to the latter author, the fatty acids, split off in the digestion of neutral fat and practically insoluble at the slightly acid reaction of the intestinal contents, are brought in solution and made diffusible by the hydrotropic action of the bile salts. The prominent rôle of these compounds in fat absorption, even in human beings, is now supported by much additional evidence. Thus, Papenkort observed that the administration of a fatty meal is followed by a marked decrease in the excretion of bile salts, probably because they are retained by the absorbed fatty acids. Moggi, in three cases of congenital total atresia of the bile duct, found that from 28 to 55 per cent of the ingested fatty acids were excreted

<sup>2</sup> Many other papers have been published recently, dealing with the characteristics of the action of the lipases, including those of the digestive glands. But, as most of these experiments were carried out with extracts of organs and with substrates that do not generally occur in food, the results, however important they may be from a theoretical point of view, cannot directly be extended to the natural digestion of fats. As pointed out by Bamann & Mukherjee, their practical bearing is restricted to establishment of the conditions which must be realized in order to obtain a more effective substitutive therapy, when pathological deficiency of the lipolytic enzymes is suspected.

in the feces. Since the ratio of the soap-free fatty acid fraction to the neutral fat was quite normal, it was argued that only absorption, and not digestion, was impaired by the lack of bile in the intestine.

However, as shown some years ago by Verzář & Kúthy (1929) and by Fürth & Minnibeck, the amount of bile salts which is daily secreted, or which is actually found in the intestinal content, is far less than would be required to explain the solvation of the large quantity of fatty acids readily absorbed under physiological conditions. Consequently, some other factors must play additional rôles in the process.

Verzář suggests that the highly surface-active bile salts, adsorbed by the intestinal cells, can act specifically in altering the intestinal permeability for fatty acids.

An action of lecithin in increasing the solubility *in vitro* of the fatty-acid bile-salt complexes, especially in the presence of small amounts of inorganic salts, was claimed by Fürth & Scholl and by Fürth & Minnibeck, but their results were disproved by Szörenyi and Müller. This discrepancy may be explained by the later experiments of Fürth, Breuer & Herrmann, who showed that the lecithin effect is obtained only in strictly determined experimental conditions. But, as the phenomenon appears to be a very complicated one, any extension to physiological fat absorption of results afforded by diffusion experiments *in vitro*, must be regarded as quite arbitrary.

The unsaturation of the fatty acids may be also an important factor in their absorption. The solubility *in vitro* at 38° and different pH's of some naturally occurring fatty acids was lately reinvestigated by Erödi. While stearic and oleic acids were soluble only in alkaline media, linoleic, linolenic, and arachidonic acids were quite soluble even near neutrality. As the surface tension of fatty acid solutions increases with increase of iodine number, it is argued that the more unsaturated a fatty acid is, the more finely dispersed it is, and therefore, presumably, the more diffusible. Furthermore, in the presence of highly unsaturated acids, even oleic and stearic acids become soluble at pH 7.3.

The greater solubility of the unsaturated fatty acids can account for their preferential absorption by the intestine. In children, Holt, Tidwell & Kirk found that unsaturated fats were more easily absorbed than milk or saturated fats, the difference being especially evident in premature infants. Fecal fats were always much more saturated than those taken in with food, and as the amount of ex-



creted fat would be too slight to account for the observed differences, it was concluded that unsaturated fatty acids are much more readily absorbed.

On the other hand, it is quite possible that even saturated fatty acids are partly desaturated in the intestine. Earlier investigators have shown that desaturation does occur when fatty acids are incubated with a mixture of pancreatic juice and bile. In the latter, a dehydrogenating enzyme for stearic acid was recently found by Quagliariello (1), while its presence in pancreatic extracts was claimed by Berend (1933), but disproved by Mazza & Stolfi (2). In conclusion, there are many plausible possibilities by which the well-known hydrotropic action of bile salts may be enhanced and completed, but how far any of the related factors is really essential in bringing about the physiological fat absorption, will be the task of future investigators to determine.

Concerning the mechanism by which the absorbed fatty acids are resynthesized into neutral fat, the possibility of an intermediate formation of phospholipids was suggested, some years ago, by Sinclair (1929). More recently, Verzár & Laszt (1) have shown that, when oleic acid and bile salts were introduced into a loop of rat intestine, absorption took place at a fairly constant rate, which was not affected by the addition of glycerol or phosphate alone. On the contrary, when glycerol and phosphate together, and much more when glycerophosphate, were added to the oleic-acid bile-salt mixture, absorption was markedly improved, the mean increase amounting to 150 per cent in the latter case. If the rats were previously injected with monoiodoacetate, no fatty acid was absorbed. In later experiments [Verzár & Laszt (2)], it was found that the absorption of olive oil, given *per os*, was likewise totally abolished by monoiodoacetate, as well as by phlorhizin. Since both of these substances are known to inhibit the esterification of the hexoses with phosphoric acid, it was supposed that the absorption of fatty acids is dependent upon an analogous phosphorylation process, which is equally impaired by phlorhizin or monoiodoacetate. Further evidence on this point was afforded by Süllmann & Wilbrandt, who found that phospholipids greatly increase in the intestinal lymph of rabbits during neutral fat absorption. The increase is roughly parallel to that of the neutral fat, the latter fraction forming, even in the course of fat absorption, about four-fifths of the total fatty acids in the intestinal lymph. This fact seems to indicate that the greater part of the phospholipids, syn-

thesized in the intestinal mucosa, are retransformed into neutral fat when discharged into the chyle.

In the papers reviewed above, it is implicitly admitted that neutral fat is fully hydrolyzed, prior to undergoing absorption, the older view of an absorption of unsplit fats in a particulate form being generally rejected. However, an unsaponifiable aliphatic hydrocarbon, such as *n*-hexadecane, appears to be well absorbed in the rat (El Mahdi & Channon) and in the cat (Channon & Devine). Furthermore, Strack, feeding dogs for long periods by a duodenal fistula, observed that, while triolein and ethyl- or methyl-esters of oleic acid were well tolerated and almost completely absorbed (90 per cent or more), free fatty acids or soaps caused severe toxic symptoms and absorption did not exceed 50 per cent. Addition of glycerol or bile salts was without marked effect upon absorption or toxicity. It is therefore suggested that esters of higher fatty acids can be absorbed as such, and, as borne out by the high toxicity of free fatty acids and soaps, the splitting of neutral fat may stop at an earlier stage.

Though the greater part of the absorbed fats are conveyed by the lacteals and thoracic duct, satisfying evidence was not afforded by earlier investigators as to whether a definite portion enters the blood capillaries directly.<sup>3</sup> In the more recent experiments of Sulze, fats fed to cats, in which the lacteals had been tied off, were almost completely absorbed. No toxic disturbances were apparent; however, the absorptive increase in blood fat was delayed, and fat was abundantly deposited in the mesentery and liver.

*Sterols*.—Süllmann & Vischer found that in the intestinal lymph of rabbits, fed with cholesterol, free and combined cholesterol rise to about the same extent. Additional evidence is thus afforded in support of the earlier view that, during absorption, the free cholesterol undergoes a partial esterification. As an increase of cholesterol (especially free cholesterol) took place even when only neutral fat was fed, it is suggested that the latter improves the reabsorption of the cholesterol excreted by the upper part of the intestine. If esterification of sterols is really an essential factor for their absorption, it may be that vegetable sterols are practically not absorbed owing to their incapacity to undergo esterification (Dam & Starup).

*Cerebrosides*.—These are not absorbed by suckling infants. In older children absorption takes place to some extent; this fact is

<sup>3</sup> Cf. *Ann. Rev. Biochem.*, 1, 271 (1932).

possibly associated with bacterial digestion in the large intestine (Beumer & Fasold).

*Phospholipids.*—These substances are probably hydrolyzed very easily by the digestive lipases. However, as reported by several investigators, the phospholipid content of many tissues increases after feeding phospholipids. According to Heinlein, this increase fails to appear when the bile duct is occluded. Further work is necessary in order to ascertain the exact forms in which phospholipids, or their split-products, are absorbed and transferred to the blood.

### BLOOD LIPIDS

*Lipid-protein complexes in blood plasma.*—The present knowledge on such complexes is summarized by Rimington and by Sandor. According to the latter, the amounts of the lipids bound to albumin are fairly constant, while those retained by the globulins are widely variable in different species and different individuals of the same species. Likewise, striking specific variations exist in the tenacity of the lipid-protein bonds, since different alcohol concentrations are required to attain the maximum extraction of serum lipids. It is suggested that lipids and proteins are united through accessory valences. Lipid-protein complexes are readily formed *in vitro* by addition of emulsified lecithin to native serum, but not to sera previously treated with the alcohol-ether mixture (Went & Kúthy). From human serum the lipids are nearly quantitatively precipitated by the Folin-Wu procedure (M. E. Turner) or by saturated ammonium sulphate at pH 3.0 (Sandor, Bonnefoi & Goret).

Süllmann & Verzár found that lipids from lipemic blood diffuse in considerable amounts only through membranes which are permeable at least to congo red (the pore diameter of these membranes is calculated to be about  $18 \times 10^{-8}$  cm.). In the diffused lipids, the iodine number and the ratios between the different fractions are about the same as in the original serum. In the opinion of the reviewer, the unsaturated fatty acids, being bound in the protein-lipid complexes, do not show the preferential diffusibility which would be expected from the results of the experiments upon free acids (see page 201). The possibility that unsplit protein-lipid complexes can go through the capillary walls, at least under special conditions, is substantiated by the rough parallelism which appears to exist be-

tween the lipid and protein contents of some pathological fluids. Confirming this statement, Bruger also found that in the course of the ultrafiltration of pleural and ascitic transudates, the cholesterol and proteins were concentrated to about the same extent in the ultrafiltration residue. However, since cholesterol, but not the proteins, is adsorbed by a Berkefeld candle or by kieselguhr, the bond between the two substances must be a very weak one.

*Postabsorptive values.*—In human blood, all the lipid fractions increase with age (Parhon, Ornstein & Sibi). According to Monasterio (2), the blood cholesterol of young men is much more variable than the phospholipids and the neutral fat. In dogs, large diurnal variations (from 50 to 100 per cent) in the total-fatty-acid content are claimed by Munoz; on the other hand, cholesterol shows little variation. A more striking constancy of the whole-blood cholesterol was found in rabbits by Cioglia & Tore and in rats by Kooy & Rosenthal.

Technical faults are probably responsible for the discrepancies existing between these and many earlier results, while in some cases it seems to the writer that too little attention is paid to the influence of variable factors other than diet. For instance, the greater constancy of the cholesterol values found in the writer's laboratory by Cioglia & Tore is presumably dependent upon the choice of young immature rabbits, the cholesterol variations connected with the sexual life phases being thus eliminated. However, the possible existence of considerable differences in the variability of the blood-lipid level in different species cannot be denied.

Decreases in the amount and in the iodine number of blood lipids, when a fat-free diet is fed over a long period, were found again by Hansen & Burr in rats and by Williams & Maynard in goats.

*Alimentary lipemia.*—A rough parallelism between the iodine numbers of food fats and of blood fatty acids during absorption, was found in children by Wilson & Hanner. However, as is well known, alimentary lipemia is the result of many complicating factors, among which the entrance of absorbed lipids into the blood is but one. Additional evidence of this is afforded by Leites and associates, who found striking changes in the amount and distribution of blood lipids, after a single meal of fat, as well as of meat or glucose.

*Blood corpuscles as lipid transporters.*—Artom injected intravenously into dogs, thin emulsions of iodized fats. After various intervals of time, the amounts of the total chloroform-soluble, acetone-insoluble, and acetone-soluble iodine were estimated in liver, plasma,

and corpuscles. Most of the injected fats were rapidly taken up by the liver, but afterwards the lipid iodine decreased in the liver and slowly increased in the blood. A notable, and sometimes dominant, fraction of the iodized lipids was found in the corpuscles. In any case, the amount of the acetone-insoluble iodine was markedly higher in the corpuscles than in the plasma. Similar results were obtained by Artom & Peretti in later experiments, in which, after feeding iodized fats to rabbits, definite amounts of iodine were found in the acetone precipitate of the blood (and especially of the corpuscles), as well as in that of the liver. These findings not only demonstrate that exogenous fatty acids are partly introduced in the phospholipid molecule, but afford direct evidence that the blood corpuscles are largely concerned in the transport of ingested or mobilized lipids (especially phospholipids): a suggestion which was first forwarded by Bloor (1915), but upon which some doubts were cast by later investigations.

In rabbits, injected lecithin was found by Sueyoshi & Okonogi to cause an increase in the lipid phosphorus of the corpuscles nearly equal to that in the plasma. Since the maximum increase was not attained until about twenty-four hours after the injection, it may be presumed that the injected phospholipids were taken up by the organs and subsequently discharged into the blood.

An absorptive increase of cholesterol, both in the corpuscles and the plasma, was observed by Chiwachi. The increase in cholesterol esters was much more pronounced in the corpuscles, in which the ratio of cholesterol esters to free cholesterol rose from the fasting value of 0.18 to that of 2.0 to 2.5 during the cholesterol absorption.

#### DISTRIBUTION OF CIRCULATING LIPIDS IN THE TISSUES

The distribution of iodized fats, when injected intravenously into dogs (Peretti, Reale & Cioglia), was compared with that of the same fats, fed to rabbits [Peretti (1)]. In the first case, high contents were constantly found in liver, spleen, and bone marrow, while that of the lungs was quite variable. After oral administration the iodine concentration was distinctly higher than that of the blood only in the liver. It was therefore argued that quite different mechanisms are involved in the two cases, the differences likely depending upon the different physico-chemical states of injected or absorbed fats.

These results do not support the hypothesis that the lungs aid in fixing and destroying absorbed fats. On this question, additional

conflicting data have been published. (Contrary: Francaviglia; Rordof. In favor: Oliaro; Kanitz; Jeckeln; Binet, Aubel & Marquis.) The present writer believes that the new findings, critically surveyed, cannot change the general conclusion which was drawn from earlier results by Bloor in the previous reviews:<sup>4</sup> that the processes of fat metabolism in the lungs do not differ essentially from those occurring in any other vital tissue.

Injected cerebrosides were found to be accumulated in various organs (Beumer & Fasold), especially in the spleen (Kimmelstiel & Laas). Vegetable sterols, introduced intravenously in rabbits, were identified by Dam & Starup in the liver and lungs, but not in the brain; the deposited sterols were never esterified, thus confirming earlier observations by Schönheimer and associates (1929-30).

The special rôle of the liver in taking up absorbed or mobilized lipids is further emphasized by many recent contributions. Diurnal variations in the neutral fat of the rat's liver are reported by Ohlsson & Blix, these variations appearing to be independent of the food intake, but alternate with the glycogen variations. No cyclic change was found in the amount of phospholipids or water.

Previous results upon the dependence of fatty infiltration during fasting on the amounts of fat available in the depots, are confirmed by Dible & Libman on fasted rabbits.

Peretti & Tore estimated, comparatively, the total fatty acids of the blood and liver during absorption of different fats. Slight but almost regular differences in the absorptive increase in the liver were found in connection with the iodine numbers of the fed fats, the more saturated oils causing greater increases in the liver fat. The maximum values were attained in the liver before those in the blood, a fact which is clearly opposed to the older view of the liver being a temporary depot for the excess circulating fat.

Several papers in recent years have shown the possibility of producing fatty livers by increasing the content of fats or cholesterol in normal diets fed to rats. In this connection, earlier results were recently completed and extended by Best, Channon & Ridout. The fatty liver caused by a high-fat diet is occasioned entirely by an increase in the neutral fat fraction. That caused by cholesterol feeding is characterized by an increase both of neutral fat and cholesterol esters. Free sterols and phospholipids are only slightly

<sup>4</sup> *Ann. Rev. Biochem.*, 1, 287 (1932); 3, 179 (1934).

changed in amount.<sup>5</sup> Choline administration, which was already known to prevent fat infiltration into the liver of depancreatized dogs, was likewise found to inhibit the abnormal changes of the liver in rats kept on a high-fat diet, as well as in those fed with cholesterol. However, the base was clearly more effective in preventing an increase of neutral fat than of cholesterol esters. As shown by analyses of other tissues, choline is likely limited in action to the lipids of the liver.

#### FAT STORAGE AND MOBILIZATION

Space is lacking to detail the results of many recent analyses of depot fats in various animals, such as those of Lovern in fishes, of Klenk (1) in frogs, of Hilditch, Jones & Rhead in fowls, of Dean & Hilditch in pigs, and of Cuthbertson & Tompsett in human beings. By comparison of these and earlier works [see also Klenk (2)], support is given to the general conclusion that the formation of depot fats is a highly selective process, which depends primarily on the biological species, and only subordinately on the temperature and on the dietary fats. The relative importance of these factors was directly proved by Zummo, on rats fed with various diets and kept either at 38° or in the ice-box. The influence of the diet on reserve fat seems to be greater in birds, while a stronger selection is exercised by these in the formation of egg lipids (Cruickshank; Henderson & Wilcke; Almquist, Lorenz & Burmester).

Concerning the intimate mechanism by which the fats are transferred from the blood to the depots and *vice versa*, accumulating evidence indicates that an active part is played in these processes by the adipose cells. The accumulation of glycogen in the latter already has been mentioned in previous reviews. As the R.Q. values seem to indicate that in the isolated tissue the glycogen is subsequently transformed into fat (Scoz), the suggestion that, at least in some cases, the glucides may act as intermediate metabolites in fat deposition and mobilization, cannot be denied. However, experimental evidence upon this point seems to be very weak.

A true lipase, splitting the triglycerides of the higher fatty acids, was found in the adipose tissue (Quagliariello & Scoz). From the

<sup>5</sup> Quite similar results have been obtained by Okey with diets rich in cholesterol and by Okey, Yokela & Knock on feeding rats with egg yolk.



same, an enzyme can be extracted which dehydrogenates stearic acid, but not the esters of this acid [Quagliariello (1)].

During fasting, the iodine numbers of the fatty acids are increased both in the blood and subcutaneous tissue (Stolfi).

These results, together with the occurrence of lipolytic and dehydrogenating processes in the isolated adipose tissue (Quagliariello & Scoz), are believed by Quagliariello (2) to support the hypothesis that fat mobilization is preceded by the hydrolysis of neutral fats and by the desaturation of the split fatty acids, in order to make them more soluble and diffusible. It is considered possible that the same processes are involved in the reverse phenomenon of fat deposition.

#### CONVERSION OF FATS TO GLUCIDES

A critical survey of the literature on this question will be found in two recent reviews by Dann and Mitchell. Both these authors come to the conclusion that the evidence for the conversion of fatty acids into glucides in the animal body seems to be merely suggestive, certainly far from conclusive.

#### PHOSPHOLIPIDS AS INTERMEDIARY PRODUCTS IN FAT METABOLISM

The theory that phospholipids are concerned both in the catabolism and transport of the fatty acids is fully discussed in a splendid review on the physiology of the phospholipids which was recently published by Sinclair (2). The more outstanding data which seem to be opposed to this theory are summarized as follows: (a) The amount of the phospholipids in the various tissues (except liver and blood) is fairly constant, in spite of sudden and extreme changes in diet or general metabolism; (b) a definite relationship is not found between the phospholipid content of certain organs (such as the central nervous system) and the intensity of their fat metabolism; (c) there is an almost constant ratio between the phospholipids and cholesterol, this constancy suggesting the occurrence of a physiological antagonism, based upon mutually opposite physicochemical properties; (d) though the composition of the phospholipids can be greatly influenced by the diet, this influence is limited by the fact that the highly unsaturated long-chain fatty acids seem to be more readily taken up by the phospholipids when they are present in the diet, and very

strongly retained when no longer supplied. The present writer agrees with the conclusion of Sinclair that these arguments are of weight "for believing that the phospholipids of animal tissues fulfill some other functions than that of intermediaries in fat metabolism." However, he does not think that the attribution of other functions to the phospholipids would necessarily exclude the possibility that these compounds, or some of them, are also concerned in fatty acid catabolism. It is not to be considered unlikely that the differences in the chemical structure of the individual phospholipids and in their relative proportions in the various tissues are connected with corresponding differences in their physiological rôles.<sup>6</sup>

The same assumption seems to be plausible, even when such striking qualitative differences are not apparent.<sup>7</sup> On the whole, if the supposition is accepted that the phospholipids in certain organs are more strictly concerned with some functions and those of other tissues with some other, the facts which are put forth as definite evidence against the participation of phospholipids in fat metabolism may receive quite a different interpretation. Furthermore, some of these facts appear to be in harmony with the special rôle which is assigned to the blood and liver phospholipids by the theory of Loew (1891) and Leathes (1909). The experimental evidence upon which this theory was originally based was of course not very strong; in fact, some of the alleged positive data were recently shown to be erroneous, or susceptible of alternative explanations. Thus, the high degree of unsaturation of phospholipid fatty acids in the liver may not definitely prove that ingested or mobilized fatty acids are de-

<sup>6</sup> Thus, the significance of the liver phospholipids is probably quite different from that of blood corpuscles, from which lecithins appear to be absent, or of the nervous tissue, in which sphingomyelins, and the closely related cerebrosides, are contained in such large amounts.

<sup>7</sup> Thus, the normal phospholipid content in different muscles seems to depend primarily upon the intensity of their usual specific activity, as shown by Bloor & Snider: acute changes during muscular exercise are observed to such slight extents that they do not exceed the limits of error (Houget). On the contrary, sudden as well as chronic changes are induced with relative ease in the phospholipid content of the liver, when the normal metabolic rate is altered. Additional evidence for this statement is afforded by the finding of an acute rise during artificial hyperthermia (Cahn). Likewise, as shown by the extensive work of Sinclair [(1932) and also (1)], in the liver and muscles the unsaturation of the phospholipid fatty acids is affected to very different degrees by the nature of the fats fed.

saturated essentially in the phospholipid molecule, the fact being perhaps better explained by a preferential fixation of the more unsaturated fatty acids. Besides, the conclusion of Meigs, Blatherwick & Carey (1919) that blood phospholipids are the precursors of the milk fats, appears to be drawn from incorrect experimental data (Blackwood & Stirling) and failed to be confirmed by Blackwood and by Lintzel.

But many recent works seem to substantiate the view that the phospholipids are concerned in both the metabolism and transport of the fatty acids: (a) the temporary rise of liver phospholipids in dogs absorbing large amounts of neutral fat [Artom (1933)]; (b) the increased formation of acetone bodies or glucose when cephalin is added to the blood perfusing through the normal or phlorhizinized liver [Jost (1931)]; (c) the disappearance, at about the same time and to the same extent, of the neutral fat and phospholipids in incubated eggs (Jost & Sorg); (d) the actual demonstration that injected or ingested iodized fats are introduced into the phospholipid molecules both of the liver and blood corpuscles (Artom; Artom & Peretti); (e) the occurrence of a phospholipid synthesis in the intestinal mucosa during fat absorption [Sinclair (1929); Verzár & Laszt (1, 2); Süllmann & Wilbrandt]; (f) the effectiveness of the essential nitrogenous constituent of the lecithins—choline—in preventing fatty liver (Best *et al.*). Certainly direct combining of ingested or mobilized fatty acids with the choline-glycerophosphate radical cannot fully explain the building up of liver phospholipids, since, in these, highly unsaturated fatty acids of the  $C_{20}$  and  $C_{22}$  series are found, which are not generally contained in the diet, and which, therefore, must be actually synthesized by the organism. These peculiar compounds are also present in the depot fats of cold-blooded animals, but not in those of the homeotherms. Owing to the more intense metabolism of the latter, it may be that these long-chain acids, formed in the liver, are more readily burned, and therefore not directly deposited [Klenk (2)]. This suggestion agrees with the fact that the  $C_{20}$  and the  $C_{22}$  acids were found by Klenk & Schonebeck (1932) not only in the phospholipids, but also in the neutral fat fraction of beef liver.

In conclusion, the present writer thinks that no decisive objection can be found to the suggestion that the phospholipids of some tissues (liver, blood, intestinal mucosa) are involved in fat metabolism, and the theory has now become far more attractive, as many important

facts have lately been ascertained which are in accordance with it. However, it must be recognized that none of these facts may be considered as direct and conclusive evidence, so that further work is needed in order that a final answer may be given to this question.

#### DESATURATION AS THE FIRST STAGE IN FATTY ACID CATABOLISM

The occurrence in pig's liver of two peculiar acids,  $\Delta^{12:13}$ -oleic and a  $\Delta^{9:10; 12:13}$ -linoleic, neither of which is present in dietary or reserve fat, was interpreted by Hartley (1909) as evidence for attributing to the liver the capacity of introducing a double bond into the 12:13 position; so that, when absorbed or mobilized from the depots, the ordinary  $\Delta^{9:10}$ -oleic acid would be converted into the  $\Delta^{9:10; 12:13}$ -linoleic acid, and stearic acid into the specific  $\Delta^{12:13}$ -octadecenoic acid. However, the latter was not found by K. Turner in sheep's liver, and even in that of the pig, Channon, Irving & Smith have recently shown that at least 80 per cent of the octadecenoic acid is the ordinary  $\Delta^{9:10}$ -oleic acid; no evidence was obtained of the  $\Delta^{12:13}$ -acid, which was the only octadecenoic acid found by Hartley.

From another point of view, a strong objection against the desaturation theory was indirectly found in recent investigations which proved that the presence in the diet of some highly unsaturated acids, such as linoleic and linolenic acids, is essential for the growth of rats [Burr *et al.* (1929)], as well as for ensuring male fertility, successful gestation, and normal lactation (Evans, Lepkowsky & Murphy). These facts imply a very limited ability of the animal body to form the "essential fatty acids," one of which, at least, (linoleic acid) would be easily produced by desaturation of ordinary oleic acid in the 12:13 position.

But, despite these negative arguments, direct proof of the desaturating capacity of living structures for higher fatty acids is now available. In two papers (which appeared in 1927, but which seem not to have attracted the attention of later investigators) Lombroso & Di Frisco showed that in minced dog liver, maintained at 37°, the iodine number of the total fatty acids sometimes increased, sometimes decreased, tending toward a final constant value. The decreases could perhaps be due to the auto-oxidation of pre-existing double bonds, but, especially when the dog was previously fed with nearly saturated fats, the iodine number rose during incubation to an extent

of 30 to 50 per cent. More recently, in Quagliariello's laboratory, the occurrence of dehydrogenating processes was demonstrated by the increase in oxygen consumption, which took place in Warburg's apparatus, when stearic or palmitic acids were added to tissue slices or extracts. Since the carbon-dioxide production was not increased, the supplementary oxygen taken up must have been used, uniquely, as a hydrogen acceptor: a statement which was further confirmed by many collateral arguments. In this way, desaturating enzymes were found to be present not only, as quoted above, in the adipose cells and bile, but also in the normal [Mazza & Stolfi (1)] and fatty liver (Califano) and, to a far less extent, in the kidney [Mazza (1)]; the enzymes are only active upon free fatty acids. Negative results were obtained with pancreas, spleen, muscle, duodenal mucosa [Mazza & Stolfi (2)] and lungs (Rordof). The desaturating activity is not confined to animal tissues: in fact, by Warburg's technique, higher fatty acids are shown to be dehydrogenated by cultures of *B. coli* (Mazza & Cimmino), and, by Thunberg's methylene-blue method, a palmitic and a stearic dehydrogenase were found in plant seeds (Grande).

In further experiments, Mazza and associates used a peculiar oleic acid with a double bond in the  $\alpha:\beta$  position. This acid was not dehydrogenated, either by bacteria previously killed with toluene (Mazza & Cimmino), or by liver extracts (Mazza & Zummo), though both these materials were quite active upon stearic or ordinary  $\Delta^{9:10}$ -oleic acid. As, on the contrary, the  $\Delta^{\alpha:\beta}$ -oleic acid was vigorously oxidized by living bacteria, as well as by liver slices, it was argued that enzymatic dehydrogenation does occur only at the expense of the  $\alpha:\beta$  bond, the later stages of fatty acid oxidation being carried out by different agents (which are probably more strictly connected with the integrity of cell structure). If the suggestion should be confirmed that the desaturating capacity is usually confined to the  $\alpha:\beta$  bonds, the present writer believes that the facts which were related above against the desaturation theory would lose significance. Furthermore, any difficulty in connecting this theory with that of  $\beta$ -oxidation would be avoided by the additional assumption that the breakdown of the fatty-acid chain normally occurs only at the  $\alpha:\beta$  double bond.<sup>8</sup>

<sup>8</sup> It may even be postulated, not only that the more unsaturated acids are not broken into multiple fragments (some of which would be odd-numbered fatty

OXIDATION OF THE FATTY ACIDS<sup>9</sup>

*$\beta$ -Oxidation.*—Quastel & Wheatley, using Warburg's technique, have tested the oxidation by tissue slices of normal lower fatty acids. With the liver, all the acids with an even number of carbon atoms (but not the odd numbered) gave rise to acetone bodies. These were also formed by oxidation of the unsaturated crotonic and isocrotonic acids. Acetoacetic acid is apparently the final oxidation product in the liver, as it was not broken down to acetone and carbon dioxide. Brain did not oxidize butyric or crotonic acids. Kidney slices oxidized these acids, but scarcely any acetone bodies were formed. The integrity of the cell organization seems to be required, as the tested fatty acids were not oxidized by minced liver. According to Mazza (2), this conclusion is applicable only to acids with less than 12 carbon atoms, the higher fatty acids being vigorously oxidized by liver extracts as well as by liver slices. It is suggested that the higher acids, owing to their colloidal state, would more easily absorb the oxidizing enzymes.

Concerning the intimate mechanism of  $\beta$ -oxidation, Butts found recently that the acetone-body excretion by rats, fed with butyric, caproic, or caprylic acids, was about the same as after feeding acetoacetic acid; a similar partition of the acetone bodies was observed. On the contrary, when  $\beta$ -hydroxybutyric acid was administered, the absolute and relative amounts of acetoacetic acid were considerably less. Therefore the keto acid, but not the hydroxy acid, is to be considered as a physiological stage in fatty acid catabolism. Increasing evidence, however, shows that oxidation can be brought about by totally different paths in different organs. Many years ago, Snapper, Grünbaum & Neuberg found that, while in the perfused liver only the well-known reversible reaction—acetoacetic acid  $\rightleftharpoons$   $\beta$ -hydroxybu-

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acids), but that those double bonds which in natural fats are not between the  $\alpha$  and  $\beta$  carbon atoms, are either previously saturated or are shifted to this position. These conjectures might agree with Dakin's experiments (1908-9) on the phenyl derivatives of  $\beta$ : $\gamma$ -isocrotonic and  $\beta$ : $\gamma$ -penthenic acids [see also Quick (1928); Raper & Wayne (1928)]. Further, they would explain the well-known failure to observe in the animal body a more rapid oxidation of the unsaturated acids, and also the likely occurrence, in incubated liver, of saturating processes, together with the desaturating ones (Lombroso & Di Frisco).

<sup>9</sup> Cf. also this volume, pp. 24, 83, 87, 264. (EDITOR.)

tyric acid—was detectable, large amounts of the hydroxy acid were actually destroyed by the surviving kidney. Furthermore, neither hydroxybutyric, nor acetoacetic acid, nor acetone was formed from butyric acid by the kidney. Recently, Snapper & Grünbaum perfused the isolated liver and kidney with blood, to which the phenyl derivatives of propionic, cinnamic, and  $\beta$ -hydroxypropionic acids were added. In accordance with the well-known results of Dakin (1909) on the whole animal, benzoic acid was formed from all three of these acids in the surviving liver. On the contrary, only the reaction,  $\text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH} \rightarrow \text{C}_6\text{H}_5 \cdot \text{COOH}$ , was carried out by the isolated kidney. In this organ, cinnamic acid was coupled with glycocholic acid, but not oxidized. Likewise, when phenyl- $\beta$ -hydroxypropionic acid was added to the blood, no hippuric acid could be isolated, and only small amounts of the cinnamoylglycocholic acid addition-product were found. It was concluded, therefore, that, unlike the liver, in the kidney normal acids are oxidized without the intermediate formation of unsaturated or hydroxy acids.

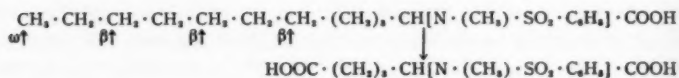
*$\omega$ -Oxidation.*—This new type of oxidation was discovered recently by Verkade and associates, who found that, on feeding the triglycerides of certain fatty acids to human subjects, dicarboxylic acids with the same number of carbon atoms were excreted in the urine. After administration of different triglycerides the intensity of the "diaciduria" was very different,<sup>10</sup> but no substantial difference in effect was detectable between odd- and even-numbered fatty acids [Verkade & van der Lee (1)]. After feeding natural fats, diaciduria appeared only when "diaciduric" fatty acids (essentially, capric acid) were contained in the administered fats [Verkade & van der Lee (2)]. When tricaprin was fed, small amounts of the lower dicarboxylic acids of six and eight carbon atoms, together with sebacic acid, were found in the urine. The formation of these acids is explained by the combination of  $\omega$ - and  $\beta$ -oxidation (one-sided or two-

<sup>10</sup> The results of Verkade *et al.* can be summarized as follows:

Tricaprylin	$\rightarrow \text{HOOC} \cdot (\text{CH}_2)_6 \cdot \text{COOH}$ (suberic acid)	..... +
Trinonylin	$\rightarrow \text{HOOC} \cdot (\text{CH}_2)_7 \cdot \text{COOH}$ (azelaic acid)	..... ++
Tricaprin	$\rightarrow \text{HOOC} \cdot (\text{CH}_2)_8 \cdot \text{COOH}$ (sebacic acid)	..... +++
Triundecylin	$\rightarrow \text{HOOC} \cdot (\text{CH}_2)_9 \cdot \text{COOH}$ (undecanedioic acid)	..... +++
Trilaurin	$\rightarrow \text{HOOC} \cdot (\text{CH}_2)_{10} \cdot \text{COOH}$ (dodecanedioic acid)	..... $\pm$
Tritridecylin	$\rightarrow \text{HOOC} \cdot (\text{CH}_2)_{11} \cdot \text{COOH}$ (tridecanedioic acid)	..... -



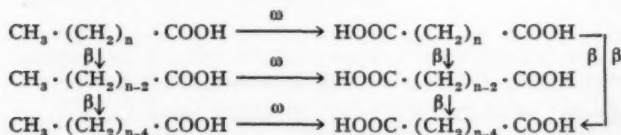
sided  $\beta$ -oxidation) [Verkade & van der Lee (3)].<sup>11</sup> Additional evidence in support of these views is afforded by the quite recent experiments of Flaschenträger and co-workers. After feeding  $\alpha$ -methylbenzosulphoamino-lauric acid to dogs, the homologous substituted adipic acid was identified in the urine. According to the authors, the initial  $\omega$ -oxidation is followed by three subsequent  $\beta$ -oxidations:



It is suggested that  $\omega$ -oxidation occurs only when the splitting of the glycerides by tissue lipases is slowed or impaired. By oxidation of the  $\omega$ -carbon atom, the union of the oxidizing enzymes with the free carboxyl becomes possible, and the main path of  $\beta$ -oxidation is then followed. This suggestion agrees with the view that the oxidation is preceded by an  $\alpha : \beta$  desaturation, occurring only when the carboxyl of the fatty acid is in a free state. (See above, Quagliariello; Mazza and associates.)

The significance of the  $\omega$ -oxidation seems to be enhanced by the fact that *in vitro*, under the action of hydrogen peroxide in the presence of a copper salt, dibasic acids (laevulic, suberic, adipic, and glutaric) are more easily oxidized than the monobasic ones. Furthermore, while the latter yield hydroxy acids as the main products, definite evidence of keto-oxidation was obtained (together with hydroxy-oxidation), when dibasic acids were oxidized. Thus, from the oxidation products of suberic acid, acetylacetone and  $\alpha$ -hydroxy derivatives were identified. From all the investigated acids, succinic

<sup>11</sup> The following alternatives are suggested:



It may be mentioned that the concept of a combined  $\omega$ - and  $\beta$ -oxidation was previously advanced in order to explain the physiological degradation of ketonic phenyl derivatives (Hermanns) or of substituted alkyl chains (Thierfelder & Klenk).

acid was constantly isolated as an oxidation product (Ponsford & Smedley-MacLean).

### KETOSIS AND KETOLYSIS<sup>12</sup>

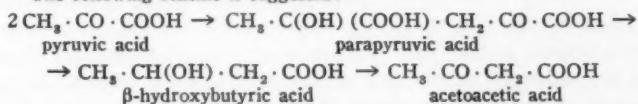
As is well known, striking differences are observed in the ease with which ketosis, especially fasting ketosis, is developed in different species. In this regard, monkeys have been shown to behave qualitatively, as well as quantitatively, like human subjects (Friedemann). In fasted rabbits a slight ketosis appears, which is increased by atmospheric depression and prevented by feeding glucides (Kallós-Deffner). In rats, a fasting ketosis is generally not detectable; but, even in these animals, a marked excretion of keto bodies is caused by phlorhizin (Goldfarb, Barker & Himwich) or by feeding acetoacetic acid [Deuel *et al.* (1933)], both effects being inhibited by a fairly definite proportion of antiketogenic substances. In this respect, galactose is far more effective than glucose; lactose has an intermediate action [Butts (2)]. Unlike the liver (Quastel & Wheatley), the blood corpuscles are found by Grégoire to transform acetoacetic acid quantitatively into acetone and carbon dioxide. The reaction is not affected by the addition of glucose, nor of glucose and methylene blue (in the presence of which glucose is oxidized to pyruvic acid). Attention is called by Annau to the possibility that the keto bodies originate not only from the  $\beta$ -oxidation of fatty acids, but also from pyruvic acid, through successive condensation, decarboxylation, and oxidation.<sup>13</sup>

### INTERMEDIARY METABOLISM OF CHOLESTEROL

No essential advance was made during the year on this problem. Complete balance experiments, previously carried out on rabbits, have

<sup>12</sup> Cf. also this volume, p. 296. (EDITOR.)

<sup>13</sup> The following scheme is suggested:



This view is based upon the oxidation of pyruvic acid by hydrogen peroxide in the presence of ammonium salts, as well as on experiments in which pyruvic acid was incubated with minced liver. In this case only keto body formation from pyruvic acid (and not from butyric acid) is increased by adding ammonium salts.

now been extended to carnivorous animals (cats). About one-half of the fed and unexcreted cholesterol is actually destroyed, the rest being deposited in all the organs except the brain. The largest deposition is found in the liver, the cholesterol content of which is increased thirty to forty times (Menschick & Page). Borgatti found that in incubated liver, spleen, and lungs, the amounts of cholesterol and cholalic acid vary almost inversely, though no quantitative correspondence could be observed between these opposite variations. This may perhaps be explained by the well-known hypothesis of the common derivation of cholesterol and cholalic acid from a unique mother substance.

#### LIPID EXCRETION

Lipids are excreted in relatively large amounts by the intestinal wall and, to a less extent, by the bile. Since only partial reabsorption takes place in the lower portion of the intestine, the fecal lipids may be essentially regarded as endogenous excretion products.

*Fats.*—Oils fed to human beings were found to cause a definite increase in the content of fats and soaps in the bile (McClure, Huntsinger & Fernald). As shown by Peretti (2) in the writer's laboratory, iodized fats, after being fed to dogs, are easily detectable in the secretion product of an isolated intestinal loop. In humans, the iodine number of the fecal lipids does not change when the iodine number of fats in the food varies from 8 to 125 (Krakower). In fasting dogs, the characteristics of the fecal lipids [previously mentioned by Sperry (1929)] are similar to those recently studied by Mortimer & Tischer in the thoracic lymph lipids; it is therefore suggested that the latter originate by partial reabsorption of excreted fatty acids.

*Sterols.*—In the bile, only free cholesterol is present (Wright) and no reabsorption takes place in the gallbladder (Varela, Vilar & Terra). However, as shown again by Wright & Whipple, the amounts excreted by the bile constitute only a small fraction of the total sterols present in the feces. In the latter, though more than 80 per cent are saturated sterols (mainly coprosterol), the presence of unchanged cholesterol was definitely proved by Schönheimer. Dam, taking advantage of the differential solubility of the corresponding digitonides in methyl alcohol, elaborated a method for the separate estimation of dihydrosterol and coprosterol. By this method, it was

shown: (a) that cholesterol is converted into coprosterol (not into hydrosterol) by intestinal bacteria, the total sterol content remaining constant during putrefaction; (b) that dihydrosterol, added to the diet, is neither absorbed nor changed into cholesterol. It is uniquely formed by the specific activity of animal tissue and eliminated as such in the feces.

*Cetyl alcohol*.—This is known to be present in large amounts in the feces. As recently shown by Schönheimer & Hilgetag, it is not present in the ordinary food nor formed by intestinal bacteria, nor excreted by the bile. If formed by reduction of palmitic acid, one would also expect to find in larger amounts the analogous compound, derived from stearic acid; however, octadecyl alcohol was rarely detected in the feces of dogs with bile fistulas.

#### ANALYTICAL METHODS

A complete system for gasometric determination of lipids in blood and tissues was recently elaborated by Kirk, Page & Van Slyke. The lipids, extracted by an adaptation of Bloor's procedure, are estimated in the Van Slyke & Neill manometric apparatus. The following estimations can be carried out in duplicate on not more than 3 cc. of plasma and with an error of not over 3 per cent: (a) total lipids (from their carbon content); (b) total and free cholesterol (from the carbon content of the digitonin precipitate); (c) phospholipids (from the ether-soluble phosphorus or the carbon content of the acetone precipitate); (d) total lipid nitrogen; and (e) lipid amino nitrogen (cephalin). Owing to the number and variety of data obtainable with greatest ease and accuracy, it seems to the writer that the proposed method should mark a decisive advance in the biochemical determination of lipids.

From a different point of view, attempts to elaborate simple and accurate procedures for the estimation of acetyl values on tissue lipids are also highly interesting. The micromethod, previously proposed by Friedrich & Rapoport, has been recently improved by Fürth, Kaunitz & Stein. Even easier and more rapid appears to be the procedure of West, Hoagland & Curtis. In this, the sample is acetylated by a mixture of acetic anhydride and pyridine and the excess anhydride is decomposed with a little water and titrated with alcoholic alkali in the presence of butyl alcohol.

A great number of recent papers deal with more or less important

modifications or combinations of well-known methods. The significance of technical "details" for the correct estimation of lipids (especially phospholipids) in blood and tissues must be fully emphasized. However, without more extensive personal experience, judgment of the actual value of any recently suggested procedure is very difficult; so that the choice of the papers, which will be further mentioned, must be regarded as rather arbitrary.

According to Mitolo, by the Fränkel extraction method, and by the additional use of adequate solvents, the different lipid fractions can be oxidimetrically estimated on very small amounts of central nervous tissue. In the analytical systems proposed by Monasterio (1) and by Katsura, Hatakeyama & Tajima, the digitonin precipitation of cholesterol is combined with slight modifications of Bloor oxidimetric procedures.

Favorable opinions on the use of the Liebermann reaction in the cholesterol estimation are again expressed by Šilink and by Bloch. Contrary to the accepted view, Reinhold found that the reaction gives higher values with plasma or pure cholesterol esters than with pure, free cholesterol; the differences disappear after saponification. Therefore, previous saponification of the blood not only insures a more complete and rapid extraction, but leads to more constant and regular results in the total cholesterol estimation. From his own personal experience, the present writer agrees fully with this statement. Furthermore, he believes that values for combined cholesterol should be better obtained by difference between the colorimetric data for free and total cholesterol. Schönheimer & Sperry applied the Liebermann reaction directly to the digitonin precipitate; Bernouilli's reaction, employed by Obermer & Milton (1933), is discredited. The superiority of the method of Schönheimer & Sperry over all the existing procedures, with respect to rapidity as well as in regard to the small amounts of blood required, is emphasized by Wasitzky in a comprehensive review on the estimation of cholesterol in biological media.

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## THE METABOLISM OF AMINO ACIDS AND PROTEINS\*

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### AMINO ACIDS

*Tryptophane*.—Matsuoka, Takemura & Yoshimatsu found that in rabbits *dl*-tryptophane forms far less kynurenic acid than does *l*-tryptophane, and concluded that *d*-tryptophane does not go over to kynurenic acid in the organism (1). Berg & Potgieter remark that the formation of kynurenic acid and the metabolism of tryptophane, with respect to growth promotion, probably follow distinct paths. They demonstrated that *dl*- and *l*-tryptophane have about the same effect on the growth of rats (2). Indeed, du Vigneaud *et al.* (3) and Berg (4) observed that *d*-tryptophane, in the isolation of which both were successful (3, 5), was also quite as effective as the *l*-form. In an earlier paper, Yoshimatsu (6) stated that in the nutrition of mice *dl*-tryptophane is inferior to *l*-tryptophane. We have, therefore, tested both experiments under similar conditions (7), using casein hydrolysate as well as gelatin as the basal diet for rats, and gelatin for mice. We were able to confirm the results of Yoshimatsu for mice, and, on the other hand, those of the American authors for rats.

Bauguess & Berg (8) selected the indole derivatives  $\beta$ -3-indoleacrylic acid,  $\alpha$ -oxymino- $\beta$ -3-indolepropionic acid, and *l*- and *dl*- $\beta$ -3-indolelactic acid for growth experiments on rats. In agreement with an earlier communication of Ichihara & Iwakura (9), they found that *dl*-indolelactic acid produced favorable results, while all the others were ineffective. Ichihara & Nakata (10) observed that in mice *dl*-indolelactic acid will not replace tryptophane. Bauguess & Berg (11) recently reported successful replacement of tryptophane by the following tryptophane derivatives: tryptophaneamide, -monoethylamide, -diethylamide, -*N*-ethylanilide, and -anilide, in addition to tryptophane ester (12).

To return to kynurenic acid, it should be mentioned that Ellinger & Matsuoka (13) proposed a path from tryptophane to kynurenic acid through indolepyruvic acid; but this seems improbable, since in the organism *dl*-tryptophane yields indolepyruvic acid more readily

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than does *l*-tryptophane (14), while the latter produces more than twice as much kynurenic acid (1). According to Bauguess & Berg (19), *d*-tryptophane forms scarcely any kynurenic acid. Since the mechanism for kynurenic acid formation assumed by Robson (15) does not yet appear to rest on secure ground, the author (16, 17) is of the opinion that kynurenic acid formation occurs at least predominantly by way of kynurenin. The structure of kynurenin has been further substantiated by synthesis of decarboxylkynurenin (18). The growth-promoting action of *d*-tryptophane may apparently be explained by its conversion into the corresponding keto acid, and re-synthesis in the organism to *l*-tryptophane. Bauguess & Berg (8) also maintain that indolelactic acid and indolepyruvic acid are probably not normal intermediate products in the formation of kynurenic acid from tryptophane.

Asayama (20) earlier established the fact that kynurenic acid cannot replace tryptophane in nutrition. We are of the opinion that kynurenic acid is not an intermediate product of the metabolism of tryptophane, but a by-product or end-product (21), and that its physiological importance chiefly lies in detoxifying excess tryptophane or kynurenin or rendering it suitable for elimination in the urine. It is interesting to note that cats, in which kynurenic acid formation has not been detected (22), often show hematuria upon administration of tryptophane (23).

From investigations of Berg and others, acetyl-*l*-tryptophane promotes growth like *l*-tryptophane (12) and is absorbed quite as well (24). The kynurenic acid formation, however, is less than that of *l*-tryptophane (19). One could perhaps explain this as due to the slow rate of liberation of tryptophane in the organism from the acetyl combination.

Certain bacteria are able to synthesize tryptophane, either immediately or after habituation, while other bacteria cannot do so (25).

Fürth, Kaunitz & Scherf (26) found 4.1 to 5.7 per cent of tyrosine in the normal human liver and 2.2 to 3.2 per cent of tryptophane; there was no essential difference in fatty liver and cholangitis. In livers degenerated by melanosis, however, they detected a much smaller amount of tryptophane (0.6 to 1.5 per cent) in the protein of the metastatic nodules as well as in the surrounding tissue, while the tyrosine content remained nearly normal.

The quantitative determination of tryptophane may be made by the spectrophotometric method of Albers & Meyer (27). Winkler

(28) states that one can also use the Adamkiewicz-Hopkins reaction if a small amount of copper is added in carrying out the reaction.

Opinions are still somewhat divergent concerning the effect of tryptophane on anemia. Matsuo (29) reported that recovery from experimental anemia was strikingly hastened by the use of tryptophane, especially with accompanying use of copper. Alcock (30) demonstrated that, contrary to Fontés & Thivolle (31), a tryptophane-deficient diet does not produce anemia in rats, and that recovery from milk anemia is independent of the presence of tryptophane in the diet. Felix, Grassmück, Huck & Matzen (32) observed that the oxygen consumption of the bone marrow is increased by certain amino acids, and perhaps by tryptophane.

Recently it was shown by Kögl *et al.* (33) that a plant-growth hormone, hetero-auxin, which is found in urine as well as in yeast, is nothing other than indoleacetic acid.

**Tyrosine.**—After blockade of the reticuloendothelial system of the rabbit with thorium dioxide (34), it is reported that the oxidation of the purine substances became temporarily disturbed, while metabolism remained unaltered with respect to carbohydrates, lipoids, and tyrosine. These results appear to conflict with those of Kotake, Masai & Mori (35) and Okamoto, Amako & Iwasawa (36). Perhaps, however, this indicates that the results of blockade vary according to the means used and the conditions. To be sure, Tabuchi (37) was able to confirm the results of Kotake and associates after more abundant use of carmine on rabbits.

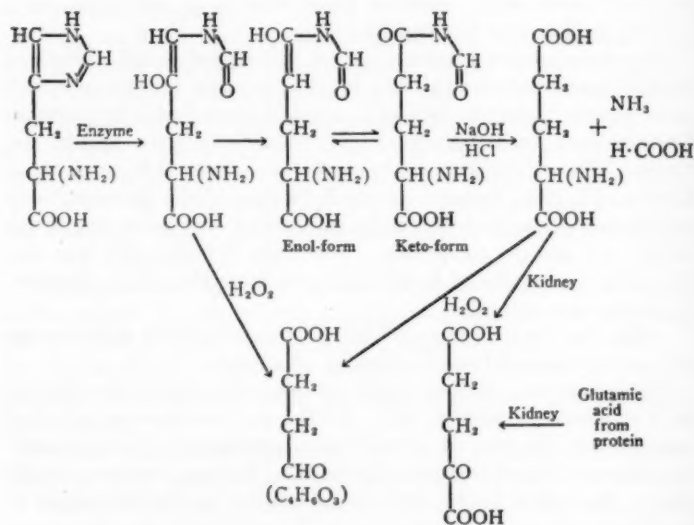
After the work of Alcock (38), it is very doubtful that tyrosine is absolutely essential in the nutrition of animals.

**Phenylalanine.**—Fölling found phenylpyruvic acid in the urine of ten feeble-minded children (39). Kotake and his students (40) had detected it in the urine of animals fed phenylalanine. Fölling named this anomaly *imbecillitas phenylpyruvica*. Perhaps, contrary to the idea of Embden & Baldes (41), phenylalanine can be deaminized in the animal organism not only by way of tyrosine but also directly as phenylalanine [compare Bernheim & Bernheim (86)].

**Histidine.**—Hirai (42) reported that a few strains of *B. coli* formed histamine from *l*-histidine. Burchard (43) found putrescine and cadaverine in the stools of dyspeptic and normal infants and of adults, and  $\gamma$ -butyrobetaine in those of normal infants, but never histamine. Kiyokawa (44) isolated from the urine of an alcaptonuric subject a substance which corresponds in properties and analytical

numbers to imidazole-carboxylic acid. It might be formed from histidine through urocanic acid; the latter, however, has not been found in the urine of this patient.

Histidase, as described by Edlbacher & Kraus (45), has received study by several other investigators (46). Edlbacher and associates (47) demonstrated by their work the formation of glutamic acid, the configuration of which corresponds with that of naturally occurring glutamic acid (48). They maintained that histidine normally is decomposed through preliminary opening of the imidazole ring, and the hitherto demonstrated urocanic acid is formed only as a by-product. Edlbacher concludes that the path of physiological disintegration of histidine proceeds as follows:



However, since Fürth & Majer (49) state that imidazole derivatives are eliminated in the urine, the hypothesis of Edlbacher on this point needs further explanation. Using Knoop's test, Kapeller-Adler (50) showed the presence of histidine in pregnancy urine. Kapeller-Adler & Kohut (51) attempted to verify this by experiments on normal, pregnant, and phosphorus-poisoned guinea pigs, and examined the

urine after subcutaneous injection of histidine monohydrochloride. They found, however, only a small excretion of imidazole bodies.

Carnosine undergoes deep-seated changes through the action of *Oidium lactis* and yeast, so that ultimately ammonia and carbon dioxide are formed (52).

*Arginine*.—In rats on a diet low in arginine, Meyer & Rose (53) found a much larger total-creatinine production than could be accounted for by the arginine of the diet.

*Sulfur-containing amino acids*.<sup>1</sup>—Absorption from the intestine decreases in the following order: cysteine, *dl*-cystine, *l*-cystine (54).

From experiments on the nutrition of rats, *dl*-cystine and mesocystine are equally able to replace cystine (55), while *d*-cystine cannot do so (56). *dl*-Cystine is oxidized in the organism less easily than *l*-cystine, but to about the same extent as mesocystine; *dl*- and mesocystine occupy, therefore, an intermediate position between *d*- and *l*-cystine (57). In the dog, the sulfur of orally administered *d*-acetylcysteine is liberated in the urine, up to 48 per cent of the dose as sulfate, and to 32 per cent as neutral sulfur (58). Stekol (59) found that dogs cannot utilize *d*-cystine.

With methionine, as with tryptophane, the *d*-form can be utilized quite as well as the *l*-form (60). By the action of nitrous acid or by means of *Oidium lactis*, there is formed from methionine the corresponding alcohol acid: in the former case the *l*-form, in the latter the *d*-form. Its zinc salt possesses a rotatory power of about  $[\alpha] = \pm 31 - 2^\circ$  (61). White & Lewis (62) demonstrated that *dl*-methionine is easily oxidized by the dog; this has been corroborated by Stekol & Schmidt (63).

In the rabbit, upon oral as well as subcutaneous administration, *dl*-methionine was oxidized in large part to sulfuric acid. Also, there occurred in the urine a substance which gave a positive cyanide-nitroprusside reaction. However, since it did not give a Sullivan reaction, it was not cystine. If one previously benzyloylated the amino group of methionine, it could no longer be oxidized in the organism. Apparently the free  $\alpha$ -amino group in methionine is essential to the oxidation of the sulfur or to demethylation. In the latter case the methionine, possibly, is converted into homocystine (64).

On the basis of experiments on a cystinuric subject, Brand, Cahill & Harris (65) concluded that the metabolism of cystine and cysteine proceed independently.

<sup>1</sup> Cf. also this volume, p. 160. (EDITOR.)



Tadpoles underwent a decided acceleration of growth in a solution of 0.001 per cent cystine; cysteine had no such effect (66).

*Glycine*.—The amount of alcohol absorbed into the blood after feeding of alcohol is decreased by absorption of glycine and alanine, as well as by protein ingestion (68). The hypercoagulability of the plasma after a meal appears to be dependent upon the concentration of the amino acids in the plasma. In the rabbit it is increased on subcutaneous injection of glycine in an amount of 2 to 4 mg. per kg.; on the other hand it is reduced on injection of 500 mg. per kg. (69).

Schmitt (70) obtained an improvement in myasthenia gravis<sup>2</sup> by administration of glycine and ephedrine sulfate. Reinhold *et al.* (71) treated eight patients who had progressive muscle atrophy with glycine with little success. According to Thomas (72), the proper field for treatment with glycine is in all primary diseases of striated muscle.

Gneiting *et al.* assert that functional examinations of the liver with glycine may be carried out satisfactorily in the presence of galactose simultaneously administered (73).

Lieben & Getreuer (74) report that a mixture of yeast, glycine, and acetaldehyde, agitated by a stream of oxygen, evolves more carbonic acid than it would without the glycine. The yeast also increases in amount, but not if the acetaldehyde is omitted. *d*-Alanine, *dl*-phenylalanine, and sarcosine have the same effect as glycine, while *dl*-alanine and proline do not.

*Alanine*.—On administration of lactic acid to young rabbits of the same litter, Hodgson (75) found glycogen formation in the liver but not in the muscle, while *dl*-alanine produced no increase of either muscle or liver glycogen.

*Aspartic acid*.—Virtanen & Tartanen (76) obtained from a bacterial mass an aspartase extract, which synthesized aspartic acid from fumaric acid and malic acid. Glimm & Nitzsche (77) assume that upon fermentation in neutral or acid solution, asparagine is changed as follows: asparagine → malaminic acid → malic acid, while in alkaline solution the following changes occur: asparagine → aspartic acid → oxalacetic acid → acetaldehyde.

#### ABSORPTION OF AMINO ACIDS AND POLYPEPTIDES

There is some evidence conflicting with the previous conception of Abderhalden (78) that proteins are absorbed from the intestine

<sup>2</sup> Cf. also this volume, pp. 247, 428. (EDITOR.)

exclusively in the form of amino acids: the chyme of the intestine may contain only about 7 per cent of free amino acids after administration of protein, while after a diet of bread it might contain 20 to 30 per cent of monosaccharide. Kotschneff (79) claims that the wall of the intestine is permeable not only to amino acids but also to more complicated amino acid complexes. In the angiotomized dog, London & Kotschneff (80) observed that protein was resorbed not only in the form of simple amino acids but also in the form of higher cleavage products. After absorption, the amino acids occurred largely in the blood corpuscles, while the polypeptides on the other hand were found preponderantly in the plasma. After muscular exertion, a decrease in amino acid content was observed in the blood plasma of the portal vein, while the polypeptide content increased slightly.

#### DEAMINATION OF THE AMINO ACIDS AND ITS LOCATION

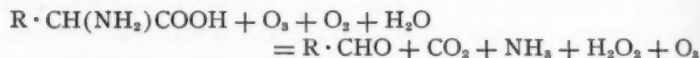
Since aminolysis by means of charcoal is not inhibited by ordinary poisoning of the charcoal, and since it also occurs with the hot water extract of the charcoal, Baur *et al.* (81) conclude that the process of deamination is not a surface phenomenon but proceeds in solution. However, since the effect is far weaker in solution, and, when ash-free, charcoal on which adsorption is heteropolar has the greatest activity aminolytically, and since the action is specific for  $\alpha$ -amino acids (82), Baur & Wunderly suggest that the aminolyzing areas are not the same as those inhibited by a narcotic (83). Subsequent tests by Wieland, Drishaus & Koschara (84) indicate that the hydrolytic deamination of alanine by animal charcoal is very dubious. Apparently the deamination involves oxygen, which is always residual in the charcoal. However, this in no way excludes dehydrogenation by the charcoal. In any case, under all conditions where ammonia was split off by the action of animal charcoal on alanine, acetaldehyde was also formed at the same time.

A glycerol extract of rose petals is able to split off ammonia from certain polypeptides more quickly than from correspondingly concentrated mixtures of the constituent amino acids. The extract, unlike kidney extract, shows negligible peptidase action, and so is adapted for direct biologic deamination of peptides (85).

Bernheim & Bernheim (86) oxidized tyrosine and phenylalanine by means of suspensions of mused liver and kidney cells, and found that the liver oxidized tyrosine readily, but phenylalanine very slowly,

while the kidney had the directly opposite effect. The oxygen consumption amounted to four oxygen atoms per molecule of tyrosine, and one oxygen atom per molecule of phenylalanine. The tyrosine was not deaminized.

Oxidation of amino acids by ozone is catalytic (87), and proceeds as follows:



In autolysis, yeast forms more amino acid in an acid medium than in an alkaline one; in an alkaline medium, conversely, it forms more nitrogen-free acids, so that in the latter case an energetic deamination is to be expected (88).

According to Aubel (89), liver and kidney deaminize alanine to pyruvic acid, while spleen, muscle, pancreas, and lung do not do so. Like results have been obtained *in vitro* (90). London, Dubinsky, Wassilewska & Prochorowa (91) observed that the deamination occurs in the kidney and particularly in the intestinal wall, with consumption of oxygen and formation of pyruvic acid.

#### UREA AND URIC ACID

Krebs (90) observed that in the organism the formation of urea proceeds from ornithine and citrulline through arginine in such a way that the liberated ornithine is again free to act catalytically. London and his collaborators (92), however, could not confirm this by work on the angiotomized dog.

Wada & Hayama (93) found that urea is formed from prolysine, citrulline, hydantoin, the ordinary amino acids, and proteins in ammoniacal solution through which is led hydrogen sulfide. Milk, blood, pancreas, and liver contain an enzyme which gives urea, *in vitro*, from the substances named (94).

Rehberg & Blem (95), in an investigation of urea formation, found that in fresh rat and frog kidney the urease method gave a higher value than the xanthidrol method, and concluded that a precursor of urea exists in the kidney. Kirk (96) states, however, that in the urease itself there occurs a substance which yields urea through the action of a kidney enzyme.

In birds, the formation of uric acid by the kidney was demonstrated with certainty in tissue slices (97). The liver formed the

precursor. Such a precursor was also demonstrated in muscle, heart, and blood; it was heat stable. In contrast with amino acids and ammonium carbonate, urea caused no increase in uric acid. On this basis, Benzinger & Krebs (98) denied the assumption that uric acid in the bird is formed from urea and a three-carbon compound. Torrisi & Torrisi (99) verified this in the bird, by showing that when propionic acid and pyruvic acid were administered with urea there was no increase in uric acid, but the urea was quickly excreted unchanged, in the urine.

### BLOOD

*Amino acids.*—Davis, Luck & Miller (100) found that insulin may be differentially inactivated, in the sense that its hypoglycemic, phosphate-lowering, and amino-acid-lowering activities may be separately destroyed. This may indicate the existence of different "active groups" in the insulin molecule. Hypoglycemia and hypoamino-acidemia may occur independently (101).

*Protein.*—The injury of liver cells by an Eck fistula causes increases of fibrinogen and globulin in the blood, as in intoxication following the ingestion of meat. This also occurs in geese on the injection of "pyripher." On removal of the liver, fibrinogen and globulin decrease, but this may be counteracted by the pyretic action of "pyripher." An extrahepatic formation of plasma protein (in the reticulo-endothelial system) therefore seems probable (102). In dogs the injection of thyroxin does not produce an alteration in the albumin-globulin ratio. The protein content is reduced only because of an increase in the water content of the blood. Thyroid extirpation does not change the plasma-protein content. Injection of bone-marrow extract increases the content of albumin as well as of globulin (103). On the intravenous injection of horse serum into dogs, there is a partial cleavage of the proteins, and partial conversion into proteins of quasi-canine specificity (104). The amino acids in normal serum globulin are not always combined in the same way. In anemia they are characteristically altered (105). The introduction of one gram of glucose per kilogram of body weight changes the protein content of the blood, which can be followed polarimetrically through hydrolysis of the serum. The course of the change in diabetics is different from that in normal subjects. Administration of 40 to 50 gm. of fat increases the effect (106).

*Blood sugar.*—When cystine is administered to dogs which are in nitrogen balance, the blood-sugar content decreases as much as 23 per cent; on the other hand, total nitrogen in the urine increases (107).

*Hemoglobin and blood cells.*—A mixture of gelatin and gliadin, itself adequate, causes anemia in rats on addition of deaminized casein, which may be prevented by addition of untreated casein. In deaminized casein, therefore, there apparently is present a toxic substance which is rendered harmless by casein (108).

Copper acetate, glycine-copper, and particularly tyrosine-copper and a copper-protein compound are effective against anemia in dogs produced by bleeding; some are also effective against anemia produced by phenylhydrazine, and may act prophylactically (109).

The regeneration of hemoglobin and blood cells occurs more readily during fasting than during carbohydrate feeding, which is explained by the protein-sparing action of carbohydrates (110).

*Oxalic acid.*—Susuki (111) determined the oxalic acid content of the blood, using a very difficultly soluble cerium oxalate [Izumi (112)]. He found in human blood 3 to 4 mg. per cent, in plasma 4 to 6 mg. per cent, and in rabbit blood 2.9 to 3.5 mg. per cent. The oxalic acid content did not increase on a diet high in protein and fat, but did with green food. It was increased by the introduction of glucose into the blood, but decreased by pyruvic acid. Asparagine, aspartic acid, and *l*(+)-aminobutyric acid increased it while butyric acid and succinic acid did not do so.

## URINE

*Urinary protein.*—If urine is extracted with ether, after removal of urates, and is mixed with the same volume of alcohol, the urine proteose is precipitated. It increases in diseases of the heart and kidney (113). The albumin-globulin ratio in the urine in nephritis bears no relationship to that in the serum and is of no diagnostic significance (114). Carbohydrate in the food can be replaced by glycerol up to 41 per cent for rats, and up to 35 per cent for dogs, without especial disturbance. However, parenteral administration of glycerol produces albuminuria and hemoglobinuria in dogs (115).

*"Vacat-O."*—The subcutaneous injection into rats of hydrolysates of vegetable protein or glutamic acid produces increases in the ratios C/N, "vacat-O"/N, and "vacat-O"/C on the same day. After daily administration, the ratios drop toward normal. This is specific for

amino acids; with glycine, however, increase in the C/N ratio is not observed because of the nitrogen increase (116).

#### PROTEIN REQUIREMENT AND GROWTH

Investigations by Süsskind (117) indicate that ingestion of one gram of protein per kilogram of body weight is not enough for the maintenance of efficiency. If up to 70 per cent of it is replaced with a biologically high-valued protein, still it does not suffice. The poor condition caused by protein-deficiency is not ameliorated by addition of fat and carbohydrate. In the experiments of Rose *et al.* (118), rats showed no growth when fed on a mixture of nineteen amino acids. Growth, however, was restored on addition of 5 per cent of the butyl alcohol extract of casein hydrolysate. The active substance is water-soluble, difficultly soluble in methyl alcohol, and forms a water-soluble carbamate and copper salt (119). Canavanine shows a favorable effect on nutrition (120), so that in rats satisfactory growth and a decrease in the food requirements are obtained.

With respect to physico-chemical constants, the protein of peas is closely related to milk casein (121) and possesses, correspondingly, the same biological value as casein in rat nutrition (122). Proteins from wheat and from yellow and white maize (123) possess about the same nutritive value for young rats, on nine weeks' use at a level of 9 to 10 per cent. The ratio,  $\frac{\text{weight increase (in grams)}}{\text{grams of protein ingested} - 10}$ , remains nearly constant. The number 10 is independent of growth and is supposed to correspond to the maintenance requirement. The value of the ratio was 1.85 for wheat proteins and 1.73 for maize proteins, and apparently shows that the proteins of wheat and maize are of biological value for growth. The biological value of raw beef for rats decreases from 67 to 60 on heating at 85°. On heating under 7 kg. pressure it is reduced to 56 (124).

#### TOXIC ACTION OF AMINO ACIDS AND PROTEINS

The administration of certain amino acids frequently injures the organs, especially the kidneys, so that a renal inflammation is observed. This circumstance, however, is variously affected by the manner of administration and the relationship of the amino acid to the other foodstuffs given at the same time (125). This is also true of

proteins. Wilson (126) observed renal hypertrophy on thirty to thirty-nine day high-protein diets, especially on use of gelatin. Bell (127) found that the subnormal rate of growth of rats on a diet of 70 per cent caseinogen was more or less improved by yeast, depending on the quantity added. On this diet alteration of the kidneys was not apparent within six months; 20 per cent of coagulated egg-white checked growth; on the introduction of 66 per cent of albumin the animals often succumbed to nephritis. At the same time there was excessive putrefaction in the intestine. According to Prunty & Roscoe (128), the renal hypertrophy of rats on high-protein diets is independent of vitamin B<sub>2</sub>. Dermatitis caused by a lack of B<sub>2</sub> is not influenced by a high-protein diet. Paunz (129) found, on injection of tyramine into dogs and rats, nephrosclerosis, which is also observed in men.

White rats on being fed diets which were protein-free, protein-free supplemented with gelatin, and protein-free supplemented with gelatin and tryosine, all showed degenerative alterations of the organs of internal secretion (130). Bong, Hilgenberg & Junkersdorf (131) observed that exclusive ingestion of proteins and peptones by dogs causes an increase in the liver weight, a decrease of glycogen, and an increase in the fat content of liver and heart, with an increase in the size of the pancreas and kidneys.

#### CALORIGENIC ACTION OF THE AMINO ACIDS AND OF PROTEIN

Lewis & Luck (132), with a special apparatus for the study of respiratory metabolism (133), investigated the calorogenic action of glycine in rats. They found a metabolism increase in fasting male rats, upon subcutaneous as well as oral administration of the amino acid. However, on subcutaneous administration in higher concentrations, the action was finally no longer detectable. With still higher concentrations a toxic effect appeared. Upon oral administration the effect on metabolic rate increased with increase of dosage up to 0.55 gm. per kg. of body weight, depending on the quantity administered. The monosodium salt of glutamic acid, administered in quantities of 0.27 to 1.2 mM (0.2 to 0.9 gm. per kg.), showed its maximum effect in doses of 0.7 mM; at this level 0.8 extra calorie per mM was given off (134). In this range, therefore, glutamic acid is a more strongly stimulating agent than glycine. Wilhelmj (135) administered different amounts of glycine and alanine, subcutaneously as well as



orally, to forty-two dogs in approximately the same nutritive state. The specific dynamic action, expressed in calories per millimol of glycine and alanine deaminized, was practically the same for both, that is, 0.2 Cal. for glycine, and 0.19 Cal. for alanine.

Aszódi & Pélyi (136) investigated the specific dynamic action of horse flesh and cooked egg-white. They found it to be related to the nitrogen content as well as to the quantity given. According to Azakáll (137), the basal exchange in quietly resting dogs in a period of eight to fourteen hours did not show a rectilinear course. Also on intravenous injection, the specific dynamic effect was independent of the quantity and kind of the introduced amino acids, so that it was thought to be dependent on a reactivity of the organism. Glutamic acid did not always show a specific dynamic effect, while alanine always increased the metabolic rate. Breton & Schaeffer published similar results (138); on the administration of peptone to the rabbit they could not establish a constant relation between the amount of the substance administered and the specific dynamic response. The fluctuations in the values obtained did not show definite connection either with the length of the previous fasting period or with carbohydrate or fat. They, therefore, believe that this phenomenon is not an expression of a quantitative thermochemical law. Borsook & Keighley (139) state that in man the catabolized protein is largely of endogenous origin, and the specific dynamic effect of the protein occurs in part through a constant factor (nitrogen metabolism), in part through a variable factor (metabolism of the deaminized residue).

The specific dynamic action of proteins as well as of amino acids may be either greater or less than normal in pathological conditions (140).

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## THE METABOLISM OF CREATINE AND CREATININE\*

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In preparing this review, we have excluded from consideration articles dealing with the chemistry of creatine and creatinine, the derivatives which they yield, and the methods involved in their quantitative estimation, except insofar as the information contained in such papers has a direct bearing upon metabolism studies. We have also omitted all references to phosphocreatine inasmuch as this substance, and its physiological functions, are adequately discussed elsewhere in this volume.

A considerable portion of the paper is devoted to the various forms of creatinuria, inasmuch as in our former review (Rose) available space did not permit a discussion of these interesting metabolic peculiarities. Because of this necessary omission, we have included in the section on creatinuria a number of papers which appeared prior to 1933, but after the publication of Hunter's monograph.

### THE DISTRIBUTION OF CREATINE AND CREATININE

Contrary to the well-known fact that creatine is a constant component of the muscles of mammals, birds, reptiles, amphibians, fishes, and certain lower forms of animal life, apparently it is not a constituent of the crab, *Astacus fluviatilis* L. Broude was unable to detect it in the aqueous extracts of a large quantity of crab meat. In the species in question it is replaced by arginine. The latter is said to be the *dl*- form, which the author believes is present, as such, in the tissues, and not produced by racemization during the process of isolation.

Comparative studies of the creatine content of right and left ventricular muscles of the heart, in a large number of human and animal specimens (Seecof, Linegar & Myers), show that the concentration in the left ventricle is greater than in the right. When the creatine content of the voluntary muscles exceeds approximately 400 mg. per cent, which is regarded as the probable saturation point, corresponding ele-

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variations in the cardiac muscles occur. According to Cowan, experimental cardiac hypertrophy in rats does not lead to increase in the actual amount of creatine present in the ventricular muscles. Thus the quantity per unit of weight in the hypertrophied tissue is about 26 per cent lower than in the normal. The hypertrophy was induced in growing rats by the production of nutritional anemia. The author points out that this condition may not be entirely comparable to types of so-called "compensatory" hypertrophy.

The occurrence of creatine and creatinine in the blood has been investigated by Bohn & Hahn. According to these authors, creatinine is not present in normal blood in measureable amounts. Also creatine is said to occur in much smaller quantities than the usual colorimetric methods indicate. The "true" creatine content is represented as varying between 0.5 and 0.8 mg. per cent. Higher apparent values are attributed to other chromogenic materials (pseudocreatine) formed by the action of hydrochloric acid and heat on the deproteinized blood filtrate. The data of Bohn & Hahn have been criticized by Zacherl & Lieb on the ground that the analytical procedure employed by the former does not accomplish a quantitative transformation of creatine into creatinine. By the use of a Pulfrich photometer, with certain other modifications in technic, Lieb & Zacherl claim that values may be secured which are more reliable than those obtained by the usual colorimetric application of the Jaffé reaction. Folin, however, has recently pointed out that the procedure of Lieb & Zacherl also is open to question in several particulars. Evidently, the most desirable method of estimating creatine and creatinine in blood is still a debatable question.

It has been recognized for many years that in nephritis the apparent creatinine content of the blood may rise enormously. Indeed, determinations of creatinine have been employed extensively for prognostic purposes. For some time it was thought that values above 5 mg. per 100 cc. of blood indicated an early fatal outcome of the disease. Many exceptions, however, have been observed. A remarkable case of retention with recovery was reported recently by Selman & Linegar. During an attack of asthma, a woman showed, temporarily, a maximum blood creatinine of 15.4 mg. per cent. After hospitalization for two or three weeks, the patient completely recovered. The blood creatinine at that time had returned to normal. Higley & Bowman report two cases in which the blood creatinine, shortly before death from uremia and terminal bronchopneumonia, reached 29.3 and 35

mg. per cent, respectively. The authors regard the estimation of this substance as an important aid in determining the prognosis in cases of chronic renal insufficiency.

In obstructive lesions of the urinary passages, such as occur in prostatic hypertrophy, the blood creatinine may be as high as in nephritis (Cantarow & Davis). After bilateral nephrectomy, the creatinine of the blood rises quite rapidly (Rathery, Dérot & Bataille). The latter authors report that in experimental nephritis the increases are less regular. In general, no change is observed during the first few days, after which a gradual rise takes place. Obviously, the rapidity of the increase in creatinine depends upon the extent and severity of the renal lesion.

In normal individuals the preformed creatinine of the cerebrospinal fluid averages about 73 per cent of that of the blood serum, as measured by the intensity of the Jaffé reaction (Maydell). In nephritis both creatinine and creatine increase in the spinal fluid, but at a slower rate than in the blood (Maydell; Gavrilá). During pregnancy the concentration of creatinine and creatine in the serum shows little change from the normal. At the beginning of the period of gestation the creatine is said to be somewhat decreased, and to undergo a moderate increase commencing a few months before, and continuing through, the puerperal state (Kessler & Albers).

Stacey finds that following the administration of creatine, the inorganic phosphates of the plasma tend to fall as the creatine content rises, but that a quantitative relationship does not exist between the decrease of the former and the increase of the latter. Incidentally, the same author finds that, contrary to other reports in the literature, the ingestion of creatine has practically no effect upon the blood sugar of normal or diabetic patients.

Gulewitsch reports the isolation from muscle tissue of methylguanidinooxalic acid or creatone, and suggests that the reversible reaction,  $\text{creatine} \rightleftharpoons \text{creatone}$ , may participate in the oxidation-reduction processes of the organism. Although the author was aware of the fact that creatone was prepared sixteen years ago by the oxidation of creatine with mercury salts (Baumann & Ingvaldsen), and that he employed such salts in separating the muscle extractives, nevertheless he concludes that the substance existed preformed in the tissue. Recently, Mann has refuted the claims of Gulewitsch by showing that the actual procedure used by the latter yields creatone from creatine.

## THE ORIGIN OF CREATINE

There still exists an amazing number of theories regarding the origin of creatine. Investigators of the problem are not even in agreement as to whether the substance is an anabolic product, the formation of which is limited to the physiological requirements of the organism; or is a catabolic substance, the production of which is determined by the intake of one or more exogenous precursors. It is impossible at the present time to harmonize these views, and the reviewer can merely outline the divergent opinions as fully as available space permits.

Arginine is still regarded by some as the source of creatine or creatinine, or both. According to Takahashi, the injection of arginine into fertile eggs, followed by their incubation, leads to a slight increase in creatinine. Injection of several other protein components is without effect upon the creatinine content of the eggs. Shapiro & Zwarenstein report that the parenteral administration of either arginine or histidine to adult male rabbits gives rise to increases of 10 to 40 per cent in the output of urinary creatinine. Under the same conditions, changes in the creatine excretion are not regarded as significant. On the other hand, the feeding of guanidinoacetic acid does not exert an appreciable influence upon the creatinine output, but induces large increases in urinary creatine. The feeding or injection of glycine, alanine, cystine, tyrosine, and glutamic acid are said to be without effect upon the output of either creatine or creatinine. The authors suggest that "the transformation of arginine and histidine into creatinine takes place in the muscles probably via creatine and that the glycoamine-creatine change is a direct one taking place in the liver." Adequate proof of this theory is lacking.

The production of total creatinine (creatine plus creatinine) in the organism of the growing rat certainly is not dependent upon the consumption of preformed arginine in the food. By comparing the arginine intake upon an arginine-low diet, and the formation of creatine and creatinine, as measured by the increment in tissue creatine incidental to growth and the output of total creatinine in the urine, Meyer & Rose have shown that the synthesis of these metabolites is very much greater than can be accounted for by the arginine ingestion. This is true even when the use of arginine in the formation of tissue proteins is omitted from consideration. The data do not exclude the possibility that creatine and creatinine may be manufactured through

arginine as an intermediate, inasmuch as this amino acid probably can be synthesized by the rat.

Contrary to the idea that creatine arises from arginine, some hold that it originates in nuclear material. The papers of Abderhalden & Buadze favoring this concept were outlined in our former review (Rose). More recently, Chrometzka has presented similar data. This author finds that the administration to human subjects of nucleosides, purines, or uric acid increases the daily output of total creatinine. The reported effect of uric acid is not in agreement with the observations of Abderhalden, who found that this substance had no influence upon creatine-creatinine metabolism. Chrometzka is of the opinion that purines may be transformed by man into either uric acid or creatine (total creatinine). Unfortunately, some of his figures for total creatinine are quite irregular even before the administration of the nuclear material.

In addition to the above experiments upon normal subjects, numerous papers have appeared during the past two years involving studies of the creatine-creatinine metabolism in patients suffering from various types of myopathy.<sup>1</sup> It is well known that in certain conditions associated with marked atrophy of the muscular system, the creatinine output is generally lowered, and creatine makes its appearance in the urine in excessive amounts as compared with normal individuals of the same age, weight, and sex (Mader, Selter & Schellenberg; Sullivan & Hess). The subjects also manifest a diminished tolerance for ingested creatine [Magee (1, 2); Ley & Titeca]. Studies on myopathy have, for the most part, had to do with the effect of amino acid administrations upon the metabolism and clinical symptoms of the patients.

According to Milhorat, the administration of glycine to fourteen patients with progressive muscular dystrophy regularly induced an increased output of creatine. Ingestion of glycine and phosphate exerted even greater effects. The extra creatine output subsided gradually, and at varying rates in different subjects. Confirmatory evidence has been presented by Mettel & Slocum, Beard, Tripoli & Andes, and others. Kostakow & Slauck (1) find that the maximum creatinuria in cases of dystrophy is attained one to two weeks after the beginning of the glycine therapy, and that it returns to the original level

<sup>1</sup> Cf. also this volume, p. 428. (EDITOR.)

after about four weeks. Parallel with the increase in creatine excretion there is said to be a decrease in the output of creatinine, but the latter rises later as the urinary creatine diminishes. On the other hand, Harris & Brand observed no effect of glycine on the creatinine output in cases of progressive muscular dystrophy, except in one subject of uncertain diagnosis. The creatine excretion rose except in one or two cases. However, the subsequent drop, said by some to be associated with clinical improvement, did not occur. Mader, Selter & Schellenberg also noted the marked creatinogenic action of glycine, but stated that no alteration occurred in the creatinine coefficient. Like results were observed by Reinhold *et al.* In a few instances the creatinine excretion appeared to rise slightly, but on the whole both metabolites remained comparatively stable over long periods of time with constant glycine feeding. The gradual inversion of the ratio of creatine to creatinine reported by Milhorat, and Kostakow & Slauck (1), was not observed. Kisner, West & Key are of the opinion that gelatin, a protein rich in glycine, affords as much stimulation in pseudohypertrophic muscular dystrophy as does free glycine. The effect of this protein on creatine-creatinine metabolism was discussed in our former review (Rose).

Chanutin, Butt & Royster administered creatine to two brothers, aged nine and twelve years, respectively. Both manifested definite improvement in muscular ability after the first month of the treatment. Three months later they were becoming progressively worse.

Beard, and Tripoli & Beard have investigated the effect of administration of glycine and glutamic acid in a variety of muscular diseases. They report that cases classified as primary myopathies or myasthenia gravis show distinct clinical improvement following the administration of either of the two amino acids, except in a few cases where the disease has "advanced to the point of complete muscle degeneration." On the contrary, patients with the progressive nuclear type, although manifesting about the same increases and decreases in creatinuria, experience only subjective improvement, or a temporary arrest of the symptoms. Milhorat finds that in secondary muscular atrophy, administration of glycine does not exert an effect upon the creatine output. Indeed, he is of the opinion that feeding of glycine may be employed as a method of differential diagnosis between primary myopathies and secondary degenerations. In this connection the observation of Harris & Brand should be noted that the ingestion of 7.5 gm. of glycine daily

produces no effect on creatine excretion in neuromuscular conditions, except in those cases which fall into the group of Charot-Marie-Tooth dystrophies.

From the above data it will be seen that in primary muscular dystrophies, at least, feeding of glycine exerts a pronounced effect upon the creatine-creatinine metabolism. Less agreement exists, however, as to the beneficial results of the amino acid therapy. Mader, Selter & Schellenberg, and Beard and his associates, evidently believe that clinical improvement occurs in a large majority of cases of primary myopathy. On the contrary, Harris & Brand, Reinhold *et al.*, Sullivan & Hess, Kisner, West & Key, Schoo & Boer, and others report little or no beneficial effects from administration of glycine. Some of these indicate that the apparent improvement occasionally observed is very temporary in nature.

As to the chemical mechanism involved in the increased creatine excretion following glycine feeding, many investigators fail to state whether they believe the extra creatine is formed directly from the glycine administered, or arises by some indirect process. In this connection, Garot (5) finds that certain children with severe muscular dystrophy have a normal creatinine output and a high basal metabolic rate. According to this author (6), such cases manifest a greater specific dynamic action to proteins than do normal children of the same age. While these observations may have no bearing upon the interpretation of the creatinuria following glycine feeding, they serve to illustrate the complexity of the problem, and the many factors involved. When sufficient information becomes available it may not be necessary to assume a direct transformation of glycine into creatine in order to account for the observed influence of the amino acid upon the abnormal metabolism of dystrophy cases.

On the other hand, certain workers interpret their findings as demonstrating the exogenous synthesis of creatine from glycine. Kostakow & Slauck (2) believe that part of the glycine is transformed into creatine, and that the amino acid may also raise the tolerance of the dystrophic organism for creatine. Tripoli & Beard have formulated a theory to account for their findings, the essential features of which are the synthesis of guanidinoacetic acid from arginine (which also may be synthesized by the body), and the methylation of the guanidinoacetic acid. Thus, according to these authors, the function of the glycine is to supply the methyl group. In this connection they

state that glutamic acid is a somewhat better creatine former than glycine, and that valine and cystine are considerably more potent than either of the first two. In opposition to Beard's findings with glutamic acid, Thomas, Milhorat & Techner report that this amino acid is without influence upon the creatinuria of a patient with progressive muscular dystrophy. They find, however, that  $\gamma$ -aminobutyric acid is effective. It is of interest to note that if  $\gamma$ -aminobutyric acid should undergo  $\beta$ -oxidation before deamination, glycine would result. On the other hand,  $\epsilon$ -guanidinocaproic,  $\epsilon$ -methylguanidinocaproic, and  $\gamma$ -methylguanidinobutyric acids, each of which might be expected to yield creatine directly by  $\beta$ -oxidation, exerted no influence upon the creatine output of patients with myopathy.

#### THE RELATION OF CREATINE TO CREATININE THE EXCRETION OF CREATININE

Until rather recently most investigators have accepted the doctrine that creatinine is derived from creatine, and that both compounds are of endogenous origin when the diet is devoid of them. During the past few years, however, a diversity of opinions have arisen. Some believe that the two compounds have no physiological relation to each other. Others regard creatine only as an endogenous catabolite, while still others express the idea that both creatine and creatinine may arise in either endogenous or exogenous metabolism.

Regarding the first of these opinions, namely, that creatine and creatinine have no metabolic relation to each other, one must recall that an abundance of evidence is to be found in the literature indicating that creatine can be transformed into creatinine by the animal organism (cf. Hunter; Rose). Furthermore, Brand & Harris have called attention to unpublished experiments in which it was noted that creatinester hydrochloride, when neutralized, spontaneously forms creatinine. This behavior, they say, "might suggest that in the animal organism creatinine is formed from a creatine compound in which the carboxyl group of creatine is not free, but linked up as an ester, thioxy ester or amide." On the contrary, Terroine and his associates (7) believe that a large part of the creatinine excreted by rabbits and rats originates in substances other than muscle creatine. In a somewhat later paper (3) they affirm that creatine and creatinine probably arise from entirely different physiological processes. Furthermore, Zickel-



bein even states that creatine introduced into the organism is not changed into creatinine. Apparently, he is not aware of the important experiments of Benedict & Osterberg discussed in the former review (Rose). Finally, Thomas, Milhorat & Techner have abandoned the view that creatinine is formed in the body by the dehydration of creatine. Other statements of a similar sort will be referred to under the section on creatinuria.

Beard, Tripoli & Andes believe that both creatine and creatinine may have an exogenous origin from the amino acids of the diet. Others, who have noted no rise in creatinine following prolonged administration of glycine in cases of myopathy, evidently are of the opinion that creatine only is formed from the amino acid (cf. Brand & Harris; Kisner, West & Key). For obvious reasons it is impossible to harmonize these divergent views, and an attempt to do so at this time would be futile.

In a number of papers Terroine and his associates (5, 7) have pointed to creatinine as an endogenous metabolite. They reaffirm what has been known for a long time that, on diets essentially free of proteins, the creatinine output remains constant in pigs, rabbits, and rats. Also, growing pigs, on such diets, are said to excrete rather constant quantities of creatinine despite alterations in the output of several other urinary components [Terroine and associates (4)]. However, in investigations conducted by Mader, Selter & Schellenberg, on children receiving a meat-free diet, and varying in age from twenty days to thirteen years, the authors find that with increase in age (greater muscular mass) both the absolute amount of urinary creatinine and the creatinine coefficient rise.

For several years opinions have varied concerning the effect of muscular activity upon creatine-creatinine metabolism. According to Loewy, Eysen & Oprisescu, severe muscular work induces increased excretion of both total and preformed creatinine. They state that distinct differences are noted between periods of rest and of activity. Their experiments were conducted upon athletes engaged in winter sports. Creatine is said to account for only a very small part of the rise in total creatinine.

It will be recalled that in creatine-feeding experiments, extending over periods of five to ten weeks, Benedict & Osterberg observed that of the retained creatine (i.e., creatine not eliminated as such) approximately one-third reappeared in the urine as extra creatinine. These findings led the authors to conclude that creatinine is probably only

one of the end-products of creatine metabolism, the remaining two-thirds of the retained material being catabolized by methods which do not yield creatinine.

In this connection, two papers of interest have appeared in recent years. Kappeller-Adler & Toda find that creatine feeding causes a three- to six-fold increase in the urinary output of methylamine. More methylamine is found in the urine of the female than in that of the male. Furthermore, all conditions associated with creatinuria (diabetes, pregnancy, muscular exertion, and to a somewhat less extent, phosphorus poisoning) induce an increased excretion of methylamine. On the contrary, arginine feeding has no effect upon the methylamine output. The authors believe that methylamine, as well as creatinine, are products of creatine catabolism.

Another product more closely related to creatine, namely, guanidinoacetic acid, has been isolated recently by Weber from the urine of two patients with pseudohypertrophic muscular dystrophy. Each patient was receiving 15 gm. of glycine daily. As far as can be determined from the limited information available, one may with equal propriety regard guanidinoacetic acid as a decomposition product of creatine, or as an intermediate in the synthesis of creatine from glycine. In either event its isolation is of unusual interest.

Perhaps twenty-five or more papers have appeared during the past two years dealing with creatinine and creatine clearances. Inasmuch as the problems therein discussed relate more to renal function than to metabolism, they are omitted from this review. For further information the reader is referred to the papers of Bordley, Hendrix & Richards, White & Monaghan, Davenport and associates, and Pitts.

#### CREATINURIAS

Reference has already been made to the creatinuria observed in many types of myopathy, with and without amino acid therapy. It seemed best to discuss this aspect of creatinuria in the section dealing with the origin of creatine, inasmuch as many of the experimental findings are interpreted by the authors as demonstrating the synthesis of creatine from glycine, or some other exogenous amino acid. However, there is a variety of other well-known forms of creatinuria to which attention is now directed.

*Creatinuria of children.*—From birth to puberty creatine is a constant but variable component of the urine. This peculiarity, which has been observed repeatedly in the past, is emphasized anew by Garot (1, 2, 3, 4), Wang & Kaucher, Daniels & Hejinian, Magee (1, 2), and others. Creatine is present also in the urine of premature infants, according to Paffrath & Ohm, but during the first three weeks after birth is excreted in extremely small amounts. After four to seven months it constitutes about 10 per cent of the total creatinine output. According to Wang & Kaucher, the creatinine output of children is dependent upon the body weight, but the creatine elimination is directly proportional to the nitrogen intake. A similar view is taken by Daniels & Hejinian, who state that creatine arises from precursors present in proteins, and thus bears no relation to urinary creatinine. They regard the latter as the end-product of muscle metabolism. Similar conclusions regarding the significance of urinary creatine are held by Terroine and his associates (8, 9), as the result of experiments upon growing pigs. On the other hand, they (1) appear to differ from Daniels & Hejinian in not believing that creatinine is a measure of endogenous metabolism.

Another explanation of the creatinuria of children is proposed by Beumer & Fasold. These investigators find that very young children, who prematurely reach puberty, are able to dispose of exogenous creatine as completely as do adults. Additional data of a similar sort are presented by Fasold. The latter finds that the administration of large doses of the ovarian hormone is without influence upon the creatine metabolism of normal children. Beumer & Fasold express the opinion that sexual maturity is a significant factor in bringing about the destruction of creatine. Recently, Light & Warren have reported the finding of creatine in the urine of thirty-five of eighty-one apparently normal boys between fourteen and nineteen years of age. Unfortunately, the diets were uncontrolled. The authors state that the percentage showing creatinuria at yearly intervals remains fairly constant at the ages of fourteen to seventeen, and manifests a perceptible drop between seventeen and eighteen years of age. Only one subject of the six examined between the ages of eighteen and nineteen years excreted creatine. In contrast to the above results, Buadze finds that boys excrete creatine until six to ten years of age. Girls manifest a constant creatinuria until puberty, after which they are said to have a cyclic creatinuria. The author thinks it probable that the male sex hormone inhibits the output of creatine, and that the female hormone

promotes its appearance in the urine. Further experiments upon a large number of subjects ingesting rigidly controlled diets will be necessary in order to determine the exact age at which creatine ceases to be a constant component of the urine of children and youth.

*Creatinuria of women.*—Intermittent creatinuria is commonly observed in women. Wang, Hawks, Huddleston, etc., find that the creatine output is directly proportional to the protein content of the diet. Others have expressed like opinions (cf. Hunter), indicating an entirely exogenous source of the creatine. If one may judge from the results of a single protracted experiment, the data of Rose, Ellis & Helming demonstrate that the creatinuria of normal females is not associated with an inability to transform orally administered creatine into creatinine. Inasmuch as the female subject is as competent in this respect as the male, the suggestion was made that women may be less efficient than men in storing or metabolizing that portion of the creatine which does not yield creatinine. The female subject excreted 6 gm. of extra creatine as compared with a zero output by a male receiving the same diet and creatine intake. More recently, Mühlbock & Kaufmann state that they are not in accord with those who find a diminished creatine tolerance in normal women. However, they administered the creatine parenterally rather than orally as in the experiments of Rose, Ellis & Helming.

*Relation of the sex glands to creatinuria in adults.*—The rôle of the sex glands in the creatinuria of adult men and women has been investigated extensively during recent years. Apparently, these studies were suggested by the interesting observations of Beumer & Fasold in children. Remen (1) reports that after the intravenous injection of 0.5 gm. of creatine into normal men, practically none of the substance reappears in the urine. On the contrary, the aged, who have lost their sex function, show, under identical conditions, a pronounced creatinuria. A lowered creatine tolerance was observed also in a castrated human subject. This is in harmony with the earlier findings upon eunuchs, that emasculation sometimes leads to a continuation into adult life of the prepubertal creatinuria. The same author (2) has secured like results with aged women. After the climacteric, women excrete a considerable part of parenterally introduced creatine. Furthermore, their ability to utilize (retain) creatine is improved by previous treatment with the ovarian hormone. According to Lasch, aged men and aged women behave alike toward intravenous creatine

injections, and both are similar to children with respect to their creatine-creatinine metabolism. Confirmatory evidence is presented by Bühler, who affirms that the male and female sex hormones are sex specific in their action. All are agreed that these findings point to a close connection between sex-gland activity and creatine metabolism.

In a series of papers involving the use of rabbits, Schrire & Zwarenstein have secured results which are rather different from those upon human subjects, as outlined above. These investigators (1) find that the castration of adult male rabbits gives rise to a 25 to 50 per cent increase in the excretion of creatinine, without the appearance of creatine in the urine. Injections of testicular extracts cause the high urinary creatinine to decrease to the normal pre-castration level (2). They believe that the castration effect is an indirect one through the anterior lobe of the pituitary. In support of this conception they state that injections of anterior lobe extracts induce an increased excretion of creatinine in normal rabbits, but have no effect on the high creatinine output of castrated animals. They point out also that normal rabbits show a high, and castrated animals a low, tolerance for subcutaneously injected creatine (3). Finally, the same authors (4) find that ovariectomy of adult female rabbits leads to a 16 per cent increase in creatinine, beginning at least five months after the operation. With females the latent period is much longer, and the increased creatinine excretion is smaller than in castrated males. Injections of saline suspensions of ovaries cause a fall in the high creatinine output of ovariectomized females. Experiments are also outlined indicating that ovariectomy does not exert a direct effect upon creatinine metabolism, but an indirect one through the anterior lobe of the pituitary (4).

*Creatinuria associated with disorders of carbohydrate metabolism.* The fact that carbohydrates play an important rôle in creatine-creatinine metabolism has been known for twenty-five years or more, and has been the subject of many investigations. In recent years interesting studies have been reported by Brentano. Riesser & Brentano find that the excretion of creatine, after the administration of narcotics or ammonium chloride, is not a direct result of the decrease in alkali reserve, inasmuch as the latter is proportional neither to the urinary creatine nor to the increment in muscle creatine. They report that even in alkalosis creatine may be excreted. A closer correlation, according to Brentano (1), is found between the creatinuria and gly-

cogen content of the skeletal muscles. Indeed, all procedures which induce decreases in muscle glycogen (administration of adrenalin, caffeine, or diuretin; convulsions; carbon monoxide poisoning; etc.), are associated with creatinuria. Thus, the condition is regarded by the author as a symptom of glycogen depletion. However, the degree of creatinuria is said to depend not so much upon the absolute amount of glycogen in the muscles as upon the extent and rapidity with which it is reduced [Brentano (2)]. Furthermore, recovery from an induced creatinuria is generally accompanied by an increase in muscle glycogen. On the other hand, liver glycogen appears to have no close relationship to creatine metabolism. The author points out that the excreted creatine is not derived from the normal stores of the body, but originates in a new formation in the muscles. Whether the glycogen depletion is the cause of the creatinuria cannot be stated, but that the two phenomena are closely associated appears to have been demonstrated. Finally, Brentano (3) has determined the effect of injections of adrenalin upon the blood lactic acid and creatinuria. He reports that when the lactic acid rises, indicating the presence of muscle glycogen, no creatinuria results. If the blood lactic acid does not rise appreciably following the administration of adrenalin, the glycogen content of the muscles is low, and creatinuria occurs. Recently, Querol & Reuter have reached similar conclusions. They find that under conditions which induce a strong creatinuria (administration of thyroxine; starvation) the injection of adrenalin fails to raise the level of the blood lactic acid. The authors add that in acute creatinuria some alteration in carbohydrate metabolism may almost always be detected. This opinion is shared also by Steinitz & Steinfeld. With regard to the blood changes brought about by adrenalin, Rigo & Frey find that in dogs the injection of 0.1 to 0.3 mg. of adrenalin per kilo of body weight causes decreases in the total creatinine, creatine, and inorganic phosphates.

Other forms of creatinuria in which a deficient supply of carbohydrate may play an important rôle are fasting [Morgulis; Terroine *et al.* (1)], intoxications induced by phosphorus or phlorhizin [Brentano (1); Terroine *et al.* (2)], and diabetes (Wolff). Lack of carbohydrate may also be a contributing factor in muscular dystrophies.

The above observations, which confirm and extend the conclusions reached by others (cf. Hunter) regarding the importance of an adequate supply of immediately available carbohydrate in preventing

many types of creatinuria, acquire new significance in view of the current conception that the resynthesis of phosphocreatine from its components is accomplished ordinarily at the expense of energy derived directly or indirectly from the transformation of glycogen into lactic acid.

*Protein metabolism and creatine excretion.*—Many students of metabolism have investigated the relation of proteins to the behavior of creatine and creatinine. The earlier of these papers have been discussed by Hunter. Reference has already been made in this review to a number of papers in which the authors express the opinion that the creatinuria of women and children is associated directly with the quantity of protein ingested; or that creatine may be formed by patients with dystrophy from almost any amino acid administered to them. At this time we shall outline a few investigations conducted upon normal animals, and involving somewhat different concepts from those described above.

In a series of papers, Terroine and his associates have studied, under a variety of conditions, the distribution or partition of the minimal output of endogenous nitrogen. The experiments involved the feeding of diets practically devoid of nitrogen, but sufficiently high in carbohydrates to meet the calorific requirements. Some of these papers have been referred to in other connections. Pigs served as the subjects for many of the experiments. As would be expected, upon such a regimen the creatinine output was very constant [Terroine *et al.* (5)], and creatine tended to disappear from the urine [Terroine *et al.* (4)]. The administration of acid to pigs upon the practically protein-free ration is said to induce a creatinuria only when the amount given was sufficiently large to occasion an increased endogenous catabolism of proteins [Terroine *et al.* (6)]. The creatinuria is thus believed to be an indirect effect. Creatinine is regarded as a product of the minimal nitrogen metabolism, while creatine is supposedly derived from the excess protein disintegration induced by the acid intoxication. Thus, in the case in question, both creatine and creatinine were of endogenous origin. Concordant conclusions are reported from the same laboratory by Boy as the result of fasting studies. According to this author, creatine arises in fasting, not from the minimal nitrogen metabolism, but from the excess proteins catabolized. Creatine is also formed, she claims, from exogenous proteins. Thus, creatinine appears to be a measure of a certain minimal nitrogen catabolism, while creatine is produced when



any additional protein is utilized, whether it be of endogenous or exogenous origin.

In this connection, Pariset finds that in rabbits, during prolonged periods of inanition, the sum of the creatine bodies excreted and those found in the total organism (minus the skin) at the end of the fast, is always larger than the amount of these bodies expected to exist in the subjects at the beginning of the starvation period. Undoubtedly, therefore, creatine-creatinine synthesis occurs during the fast, at the expense of materials liberated in the degradation of tissue proteins. The fact that an actual synthesis of creatine from endogenous sources occurs during starvation was emphasized by Mendel & Rose more than twenty years ago. On the other hand, the reviewer is not yet convinced that the production of creatine can be augmented from exogenous sources beyond the physiological needs of the organism for it.

*Relation of the thyroid to creatine-creatinine metabolism.*—Hyperthyroidism is usually associated with creatinuria. In a series of 145 cases, composed of 115 of exophthalmic goiter and thirty of adenomatous goiter with hyperthyroidism, Kepler & Boothby found creatinuria in eighty-nine cases. They verified the earlier observation of Palmer, Carson & Sloan that administration of iodine reduces the creatine output. This is attributed not to the iodine *per se*, but to the general improvement in the physical condition of the patients. Eimer states that the excretion of creatine is observed frequently in cases of hyperthyroidism, and usually in normal subjects following the administration of thyroid tablets. Hedrick and Eimer have also found, independently, that in severe cases of Basedow's disease a low creatinine coefficient exists, which is inversely proportional to the increase in basal metabolic rate. Whether these findings are associated with an accelerated protein catabolism, a deficient supply of carbohydrate, or both, is not clear. However, Brentano (2) observed a decrease in muscle glycogen accompanied by creatinuria in rabbits following the administration of thyroxine.

In young children, hypothyroidism induces a decrease in, or complete cessation of, creatinuria according to Poncher, Visscher & Woodward. The creatine excretion can be restored to the usual values for normal children by giving thyroid extract. The tolerance for creatine in children with hypofunction of the thyroid is greater than for normal individuals of the same age. Furthermore, the creatinuria after thyroid therapy occurs before a significant change in either the

basal metabolic rate, blood cholesterol, or body weight is observed. For this reason, the authors believe that urinary creatine determinations in such cases may be useful in diagnosis, and in the control of thyroid therapy.

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## DETOXICATION MECHANISMS\*

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This review confines itself, more or less, to the research work done during the last two years. The earlier work has already been covered (1, 2).

As on previous occasions, the material is grouped under convenient subheadings. In addition, the question of the site of detoxication and the theories regarding the mechanism of detoxication are included.

### OXIDATION

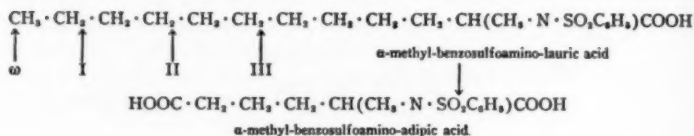
$\beta$ -Oxidation of fatty acids, according to Flaschenträger and his coworkers (3), is possible only when the oxidizing enzymes form intermediate compounds involving the carboxyl group. Sulfonic acids, for example, do not undergo such an oxidation. *n*-Octansulfonic acid fed to a dog is eliminated unchanged to the extent of 83 per cent (as shown by sulfur determinations); 40 per cent can be recovered in the form of the barium salt.

Using the more complex fatty acids, into which  $\alpha$ -amino groups were introduced, and where the  $\alpha$ -amino groups were joined to methyl and benzosulfo groups, the authors studied the products formed in the body by their ingestion. For example,  $\alpha$ -methyl-benzosulfoamino-lauric acid yields, when fed to the dog, a substituted adipic acid ( $\alpha$ -methyl-benzosulfoamino-adipic acid). Adipic acid itself contains 6 carbon atoms. The substitution product was recovered from the urine.

The explanation offered for this behavior is that "blocking" the  $\alpha$ -carbon atom prevents the usual breakdown beginning at the carboxyl end of the compound, and gives, instead, an oxidation at the *omega* carbon atom, followed by a threefold  $\beta$ -oxidation.

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In the course of their work, they found that *p*-toluene-sulfonamide is oxidized to *p*-sulfamido-benzoic acid—an oxidation of the  $\text{CH}_3$  group to the  $\text{COOH}$  group. The oxidation, as might have been anticipated, was confined to but one side chain.



Bauguess & Berg (4) have tested the ability of the animal organism to convert tryptophane derivatives into kynurenic acid. They find that the amide, the ethylamide, and the diethylamide of tryptophane, as well as the anilide and the *N*-ethylanilide, are converted to kynurenic acid and also support the growth of the animal (rabbit). Apparently, the primary, secondary, or tertiary nature of the amide is not of importance in so far as amide cleavage in the body is concerned.

Robbins (5) has made a study of the metabolism of cresols in the dog and man. (The phenols used are of value in the treatment against hookworm and ascaris.) 6-Hexyl-*m*-cresol was recovered to the extent of 4 to 8 per cent from the urine and 27 per cent from the feces. 6-Decyl-*m*-cresol (1 gm.) could not be recovered. Eighteen per cent of the hexylresorcinol was excreted in the urine and 64 per cent in the feces. The excretion of thymol (20 to 30 per cent) was confined to the urine.

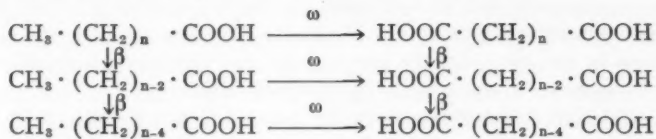
An important contribution to the subject of fatty acid oxidation<sup>1</sup> we owe to Verkade & Lee (6) who emphasize the probability of *omega* oxidation of fatty acids in the body, thereby giving rise to dicarboxylic acids. The feeding of tricaprln resulted in the appearance in the urine of large quantities of sebacic acid, as well as smaller quantities of suberic and adipic acids. The feeding of triundecylin resulted in the isolation from the urine of relatively large quantities of nonanedicarboxylic acid and smaller quantities of azelaic and pimelic acids.

Verkade & Lee are of the opinion that at first we have an *omega* oxidation of the fatty acid, with the formation of the corresponding

<sup>1</sup> Cf. also this volume, pp. 24, 83, 87, 214. (EDITOR.)



dicarboxylic acid. Simultaneous  $\beta$ -oxidations at both ends of the chain follow, forming successive dicarboxylic acids:



Kataoka (7) finds that rabbits fed with 5-vanillal-barbituric acid oxidize the substance to vanillic acid, a result which is also obtained when vanillin itself is fed. From 41 gm. of the vanillal-barbituric acid 49 gm. of vanillic acid was recovered.

Inaguki (8) finds that the subcutaneous injection of *o*-nitroacetophenone into rabbits gives rise to *o*-aminophenylethyl carbinol, while part of the substance is oxidized to anthranilic acid.

In an attempt to overcome the toxicity of piperidine derivatives, Steudel (9) condensed barbital and piperidine with formaldehyde, forming methylene-N-barbital-N-piperidine, a substance moderately tolerated. Two dogs received a total of 79.5 gm. of this substance over a period of fifty-four days. After a somewhat laborious process, the gold salt of  $\alpha$ -methyl-aminovaleric acid (1.5 gm.) was isolated. This led Steudel to conclude that methylpiperidine is first formed, hydrolysis at the point adjacent to the nitrogen of the ring takes place next, and finally the methyl group, at the end of the chain, is oxidized into the carboxyl group.

The behavior of cinnamalacetic acid, as well as the aldehyde of the same substance, has been the subject of an investigation by Friedmann & Mai (10). This acid, neutralized with sodium carbonate, was administered subcutaneously to rabbits in amounts of 4 gm. in the course of seven days. The urine was investigated, but no evidence was found of cinnamic acid, cinnamyl glycine, benzoic acid, or hippuric acid, as might have been expected. Cinnamaldehyde was administered in olive oil in amounts of 0.2 gm. per kg. body weight. In this case, cinnamic acid, as well as its conjugation product with glycine, was found in the urine. In addition, there were considerable amounts of free benzoic and hippuric acids. This is quite unusual, as most of the aromatic aldehydes are oxidized to the corresponding acids with considerable difficulty.

The fate of furanacrylic acid in the organism of the rabbit was investigated by Friedmann (11). Rabbits were given subcutaneously

0.3 gm. doses of furanacrylic acid per kg. of body weight. This had to be administered in the form of a 5 per cent solution which was neutralized with sodium carbonate. In this way a total of 3.2 gm. was administered without any ill effects. Furanacrylic acid was not found in the urine, but 54 per cent of the substance administered was eliminated in the urine as pyromucuric acid.

*dl*- $\alpha$ -Naphthylalanine was fed to dogs (12) in amounts of 10.3 gm. over a period of several days. Friedmann also fed 7.3 gm. of this same substance to rabbits by means of a stomach tube. The substance was comparatively non-toxic and was well tolerated by both the dogs and rabbits. One would expect this substance to be metabolized through a partial oxidation of one of the rings with a complete oxidation of the side chain, but such was not the case. The urine was evaporated very carefully, extracted with alcohol, the alcohol extract was evaporated and phosphoric acid added. A compound was obtained having the empirical formula  $C_{14}H_{14}N_2O_3$  with a molecular weight of 247, while the calculated molecular weight was 258. The substance so obtained was identified as  $\alpha$ -naphthylalanine-hydantoic acid. The anhydride of the acid was prepared and purified by sublimation in high vacuum and it was found to be identical with the synthetic product.

2,4-Dinitrophenol<sup>2</sup> has recently come into considerable prominence in clinical medicine on account of its power to increase cellular metabolism and subsequently to cause a reduction in body weight. Unfortunately, while the literature is crowded with clinical data regarding the effect of this compound on the body, but few investigators have interested themselves in the fate of the chemical substance. From its analogy to picric acid it might be assumed that one or both of these nitro groups might be reduced to amino groups, since picric acid is reduced in the body to picramic acid. Guerbet & Andre (13) report that at least two substances are formed in the body after feeding 2,4-dinitrophenol, one being 2-amino-4-nitrophenol and the other 2-nitro-4-aminophenol. It is suggested that such substances are always formed in the body after feeding this phenolic compound, although at times demonstrable only after hydrolysis. The authors believe such phenols to combine with glucuronic acid, or perhaps to form carbamido nitrophenols. These compounds may be found in the liver, kidney, brain, blood, and spinal fluid, as well as in the urine.

<sup>2</sup> Cf. also this volume, p. 475. (EDITOR.)

## REDUCTION

An interesting example of reduction has recently been cited by Inaguki (8). He injected subcutaneously *o*-aminoacetophenone into rabbits. Part of the substance was excreted unchanged in the urine, and part of it was oxidized to anthranilic acid, but most of the material was subjected to reduction, in the course of which *o*- $\alpha$ -hydroxyethylaniline was formed and in turn conjugated with glucuronic acid.

Heymans & Casier (14) attempted to determine the relative activity on cellular metabolism of a number of allied nitro derivatives. The compounds studied were: dinitroaminophenol, dinitronaphthol, 2,4-dinitrophenol, dinitrothymol, dinitrocresol, 2-cyclohexyl-4,6-dinitrophenol and 2-cyclopentyl-4,6-dinitrophenol, dinitro-3,5-diodothyronine, hydroquinone-2,4-dinitrophenol, 4-hydroxy-3-nitrophenylalanine, and 2-nitrohydroquinone-4-phenyl ether. The authors made no attempt to study the fate of these compounds in the animal body, but contented themselves with classifying the compounds according to the effect which each compound had in increasing cellular metabolism. The compounds were compared to 2,4-dinitrophenol, the effect of which was taken as 100 per cent.

Handovsky, Casier & Schepens (15), in studying the action of dinitrophenols as accelerators of cellular metabolism, have concluded that the intensity of the action of these different dinitrophenols is directly proportional to the speed of the action in which the nitro compounds are converted into the corresponding aromatic amines. They believe that the nitro compounds act as hydrogen acceptors and the amines as hydrogen donors. They suggest that it is probably a direct action on the cellular enzymes and also a direct action on the intermediary products of combustion.

## GLYCINE

Glycine combines with the carboxyl groups of organic acids in the process of detoxication, but the products formed seem to be limited in amounts, as various experimenters have shown. The rôle assumed by glycine may be taken over by glucuronic acid in many cases, so that there is often a question as to which one of these compounds is the more important in detoxication.

In studying the detoxication of salicylic acid, Quick (16) has emphasized a fact which has long been known to experimenters, that aromatic acids substituted in the ortho position are, as a rule, more

toxic than the corresponding meta and para compounds. Again, he has emphasized the fact that it is much more difficult to obtain a conjugation of these ortho compounds with glycine than it is with the meta and para compounds. In working with salicylic acid, Quick found that the human organism can synthesize salicyluric acid, but the amount excreted is very small and is not markedly influenced by exogenous glycine. The rate of salicylic acid excretion is dependent upon the concentration of the drug in the body. With increased doses of salicylic acid, the excretion of free salicylic acid becomes definitely greater, while the output of the glycine conjugate is only slightly affected.

The action of salicylic acid on uric acid elimination is strikingly augmented by glycine or by foods rich in glycine. Quick believes that this is not due to the specific dynamic action of the glycine, since 5 gm. of glycine had practically no effect on the excretion of uric acid, while 1.5 gm. of glycine when given with 2 gm. of salicylic acid produced a striking increase in the uric acid elimination. The salicyluric acid in this case was actually isolated from the urine.

Tulane & Lewis (17) undertook an interesting experiment in which they sought to determine the effect of the length of the carbon chain of aromatic acids on the combination with glycine. For this purpose they chose benzoic acid and phenylacetic acid and the animal employed was the rabbit. Phenylacetic acid, like benzoic acid, combines with glycine in animals other than fowls and human beings. It has always been assumed that the site of formation of phenaceturic acid (phenylacetyl glycine) is the same as that of hippuric acid, and that it is eliminated by the kidney under the same conditions.

Quick (18), working with dogs, had previously shown that when glycine was supplied exogenously the speed of conjugation of glycine with phenylacetic acid was much greater than the speed of conjugation with benzoic acid. In the dog, moreover, both of these acids are conjugated with glucuronic acid to a considerable extent. The addition of exogenous glycine, or gelatin, or proteins rich in glycine, resulted in increases in the amount of phenaceturic acid excreted, while the similar addition of gelatin to the diet increased the amount of urinary hippuric acid only slightly. With rabbits the increase in the absolute amount of hippuric acid, when glycine was fed, was much greater than in the case of phenaceturic acid, as shown by Lewis. Quick observed no excretion of free benzoic acid or

phenylacetic acid by the dog after the administration of these acids. He proposed the hypothesis that compounds of this type on introduction into the organism become "fixed," and that their eventual liberation is brought about as a conjugation product by the removal of some of the "fixed" material.

Lewis, on the contrary, observed in rabbits the excretion of considerable amounts of unconjugated benzoic acid and phenylacetic acid after the intravenous injection of these acids. When comparable amounts of benzoic and phenylacetic acids were fed, the average excretion of hippuric and phenaceturic acids in the six-hour period immediately after the feeding was equivalent to 37 and 6 per cent of the intake, respectively. After glycine was fed with phenylacetic acid, the rate of excretion of phenaceturic acid was increased, but the average total excretion even under these favorable conditions was equivalent to only 21 per cent of the phenylacetic acid. The slow excretion of the phenaceturic acid apparently is not due to delayed absorption of the phenylacetic acid from the intestine, since when phenylacetic acid is introduced directly into the blood stream the rate of excretion of phenaceturic acid is not greater than after oral administration.

Tulane & Lewis believe that the slow excretion of phenaceturic acid after the administration of phenylacetic acid is due to a limitation in the capacity to synthesize phenaceturic acid, even though an exogenous supply of glycine is available. Proof of this hypothesis is found in the fact that when phenaceturic acid itself is injected intravenously, it is rapidly eliminated through the kidney of the rabbit. Benzoic acid was found to be excreted in moderate doses within twenty-four hours, while equivalent doses of phenylacetic acid were excreted over a period of days.

Stassi (19) found that marasmus developed when sodium benzoate was administered to guinea pigs in daily doses of 1 gm. per kg. body weight. Sodium glycolate inhibited the development of this condition. It would seem, at first sight, that the inhibition might be due to the fact that the glycolate is converted into glycine and that the glycine acts as a detoxicating agent for the benzoic acid. This does not seem to be the explanation, however, for the amount of hippuric acid nitrogen contained in the urine formed only a very small fraction of the total nitrogen.

Zummo (20) repeated these experiments. He administered ammonium glycolate to guinea pigs subjected to the action of sodium

benzoate. However, he noticed that this did not arrest or diminish the endogenous destruction observed when sodium benzoate was fed. Hence he concluded that the animal organism is incapable of using either glycolic acid, ammonium salts, or both, for the synthesis of glycine.

Lombroso (21) fed sodium citrate and sodium benzoate simultaneously to guinea pigs. The animals not only maintained their normal body weight, but showed a tendency to increase in weight. He believes that when sodium citrate or sodium glycolate is fed, the beneficial results are in no way due to a synthesis of glycine with subsequent detoxication of benzoic acid, but are simply due to the beneficial results of the alkalizing influence of the sodium ion.

#### GLUTAMINE

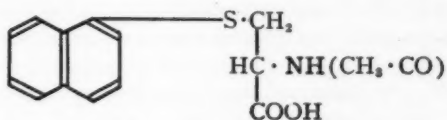
Glutamine has been found in combination only with phenylacetic acid (22), after the latter is fed to human beings. Thus far, in the literature, none of the many derivatives of phenylacetic acid fed to human beings has been known to combine with this substance. Phenylacetic acid itself, fed to various animals, combines either with glycine or with glucuronic acid, depending upon the quantity of acid fed and the type of animal used in the experiment. The exception to this is in fowls, where we find phenylacetic acid and its homologue, benzoic acid, combined with ornithine.

#### CYSTINE

The detoxication of brombenzene by acetylaminocystine leads to the formation of so-called mercapturic acid, the first detoxication product found in which cystine was used as the detoxicating agent.

Recently Bourne & Young (23) have made an interesting contribution to this mercapturic acid series. While experimenting with naphthalene, rabbits weighing between 2 and 3 kg. were kept on a diet of oats and cabbage exclusively. Naphthalene was dissolved in warm liquid paraffin and administered by means of a stomach tube. Doses of 1 or 2 gm. were given to each rabbit and repeated at intervals of two or three days. The urine was acidified and extracted with chloroform. The chloroform extract yielded a compound which upon analysis proved to have the empirical formula,  $C_{15}H_{18}O_8NS$ , with a molecular weight of 289. The compound was found to be made up of naphthalene, cystine, and acetic acid. The structure of

the compound was studied and the following graphic formula assigned to it:



1- $\alpha$ -Naphthylmercapturic acid

The structure of this new  $\alpha$ -naphthylmercapturic acid was determined by Ing, Bourne & Young (24), who synthesized the compound and found it identical in physical and chemical properties with the product isolated from the urine, thus proving it to have the structure given above.

#### SULFATE

Di Mattei (25) reports that after the oral or intravenous administration of pyrrole to rabbits there is a decided increase in the ethereal sulfate output of the urine. In a number of experiments he has demonstrated that the dose of pyrrole can be doubled, providing sodium sulfate is given at the same time. He believes this to be due to the fact that the sodium sulfate furnishes an increased supply of sulfate for the conjugation.

Apparently an attempt was not made to determine a possible conjugation of the glucuronic acid. This would lead to the conclusion that the ethereal sulfate conjugated with pyrrole is taken directly from the inorganic fraction of the urinary sulfate.

#### GLUCURONIC ACID

Pryde & Williams (26) have studied, comparatively, the conjugation of *d*- and *l*-borneol. After a careful study of these two compounds, the authors believe the dextro modification to be the more important when dealing with conjugations within the body. Hemingway, Pryde & Williams (27) have studied the site of formation and conjugation and conclude that the main, if not the only, place of conjugation is the liver. Furthermore, they found, after a very exhaustive study, that the process of conjugation is abolished by cyanide poisoning.

Pryde & Williams (28) have studied the structure of the glucuronic acid compounds. They fed borneol and isolated a compound



from the urine of human beings and animals which they believe to be the zinc salt of " $\beta$ -bornyl-*d*-glucuronide." By methylation of this glucuronide with silver oxide and methyl iodide, the 2,3,4-trimethyl- $\beta$ -bornyl-*d*-glucuronide was formed and could be obtained in crystalline form. Through the action of 0.2 *N* sulfuric acid in methyl alcohol at 100°, the compound was converted into a mixture of the  $\alpha$ - and  $\beta$ -2,3,4-trimethyl-*d*-glucuronides. Oxidation with nitric acid yielded *d*-dimethoxysuccinic acid, *dl*-oxylotrimethoxyglutaric, and 2,3,4-trimethyl-*d*-saccharolactone. The isolation of these last three products would seem to establish a pyranoid structure for the glucuronic acid residue of bornylglucuronide.

The authors (29) conclude that conjugated glucuronates (for example, benzoylglucuronic acid) are either of the glucoside type, i.e., the "ether type" of binding, or the "ester type," or perhaps a combination of these. Benzoylglucuronic acid was prepared in a high state of purity. Like all the glucosides containing a benzoyl radical, this substance is easily hydrolyzed and subsequently reduces the ordinary sugar reagents.

An interesting new glucuronic acid complex has been announced by Ichihara & Tamura (30). Tyrosinehydantoin was prepared in the usual way from *l*-tyrosine and potassium cyanate. The substance was fed in daily amounts of 5 gm. to two rabbits over a five-day period. The urine was precipitated with basic lead acetate, the precipitate decomposed by hydrogen sulfide, and the filtrate reprecipitated with mercuric sulfate in the presence of sulfuric acid. After successive treatments with lead carbonate and hydrogen sulfide (with potassium carbonate), crystals were obtained which proved to be the potassium salt of tyrosinehydantoin-glucuronic acid. The yield was 9 gm. The product was identified through hydrolysis with sulfuric acid into tyrosinehydantoin and glucuronic acid. There seems to have been little or no ethereal sulfate formed together with the glucuronic acid complex. However, subcutaneous injections of hydantoin were followed by very slight increases in ethereal sulfate.

The authors consider the glucuronic acid complex of much more importance in the mechanism of detoxication than the sulfuric acid conjugation. We have here, perhaps, a partial answer to the old question as to the relative importance of glucuronic and sulfuric acids in the detoxication of hydroxy compounds. It would seem that after the feeding of tyrosinehydantoin the animal organism used only glucuronic acid for detoxication because the substance was

slowly absorbed, while after injection the substance was more quickly absorbed and required a more rapid process of detoxication. The hydantoin of the amino acids are very stable and so far have been employed in feeding experiments, largely because both the amino and carboxyl groups of the amino acids are blocked; thus it was possible to show that an amino acid so protected could withstand the oxidative mechanisms of the body.

Another new glucuronic acid compound is reported by Inagaki (8) who, after subcutaneous injection into rabbits of *o*-nitroacetophenone, found much of the compound reduced to *o*- $\alpha$ -hydroxyethyl-aniline, which substance was, in turn, conjugated with glucuronic acid and excreted in the urine.

We still have many questions to settle regarding glucuronic acid. Quick believes that benzoylglucuronic acid is not the usual type of ether or ester binding as had been supposed, but that the benzoyl radical is attached through the secondary alcohol group, forming the glucuronic acid monobenzoate. Pryde & Williams, however, do not share this view. They remain of the opinion that the ester and ether types of binding of the glucuronic acid should be retained.

We still have to account for the origin of glucuronic acid. If we consider it to be derived from glucose, then the question arises as to whether it is a product of normal glucose metabolism or whether it is derived in some other way when detoxication and glucuronic acid formation are necessary.

Quick (31) has shown that 5 gm. of glucuronic acid can be synthesized for purposes of detoxication within a period of twenty-four hours without any increase in nitrogen metabolism. He also showed that when a glucuronogenic drug was fed to a diabetic dog, glucuronic acid was synthesized from the glucuronogenic fraction of the protein molecule. This would seem to indicate that glucuronic acid comes from glucose and not from the amino acids, as was at one time suggested. Here we have the sugar-forming amino acids first converted into glucose, and the latter used for the formation of glucuronic acid.

Quick (32) found that the action of insulin increases the output of glucuronic acid and the feeding of acetoacetic acid reduces this output. The use of insulin simply mobilizes greater quantities of sugar, thus making it available for glucuronic acid synthesis. Acetoacetic acid, or any of the acetone bodies, is first attacked by the body and is oxidized by using all the available glucose; in conse-

quence, any glucuronogenic drug fed at this time is not detoxicated until the more poisonous acetone bodies have been first removed.

Quick postulates the theory that the formation of glucuronic acid from glucose may not only be a normal process in the catabolism of the latter, but that the initial step in the catabolism of fatty acids may be a conjugation of these substances with glucuronic acid (like the combination of benzoic and phenylacetic acids with this substance).

Miller & Conner (33) simplified the problem considerably. They believe that glucuronic acid is liberated in sufficient quantities from the mucin of the gastro-intestinal tract and is readily available for conjugation with toxic substances. They also suggest that the substance is preformed in greens and in connective tissue. In fact, they think it questionable whether the rabbit can synthesize glucuronic acid from carbohydrates or from amino acids.

Miller, Brazda & Elliot (34), in experiments on dogs, find that glucuronic acid is derived from the digestion of mucin. They believe that this substance may be stored in the body, and may readily be available for conjugation. When the body is depleted of mucin, toxic symptoms will appear, due to the inability of the organism to form glucuronic acid. It is possible that glucuronic acid may be formed from the carbohydrate portion of mucin; however, this is perhaps negligible in quantity. This phase of the question was investigated by Ambrose & Sherwin (35), who failed to share the opinion of previous experimenters.

#### ACETYLATION

Acetic acid is employed in the detoxication of amino groups in the animal organism. Harrow, Mazur & Sherwin (36) found that the rabbit acetylates *p*-aminobenzoic acid to the extent of about 25 per cent after doses of 1 gm. The output is increased by the injection of insulin but is not increased by the injection of glutathione. It is known that reduced glutathione has an inhibiting effect upon the activity of insulin *in vitro*. Simultaneous injection of insulin and glutathione is without effect. They suggest that the increased acetylation after insulin injections is probably due to the increased mobilization of sugar in the organism, and that the acetic acid is produced as an intermediary product in the catabolism of the carbohydrates, and may even be formed from the  $\beta$ -oxidation of the fatty acids.

## GLUTATHIONE

The importance of glutathione in the detoxicating mechanism of the animal body has been the subject of much speculation. It is conceivable that glycine, glutamine, and cystine might easily result from the breakdown of this tripeptide. Also, ethereal sulfate may well be derived from the cystine portion of this same molecule. Glucuronic acid is somewhat more difficult to visualize as coming directly from this source.

Brand & Harris (37) have discussed the theoretical aspect of the problem. However, a number of experimenters have been working in this field for the past few years and so far have made little progress. It is necessary to combine such compounds as phenylacetic acid, benzoic acid, or brombenzene with glutathione and then to feed or inject such a combination to prove that the synthetic product is broken down into hippuric acid, phenaceturic acid, or mercapturic acid, respectively.

Such a procedure, however, seems quite impossible, as the sensitivity of the glutathione molecule is much too great to admit of such a procedure. Again, it might be possible to feed various substances such as benzoic acid, phenylacetic acid, or brombenzene, and inject glutathione, in an attempt to show that such a procedure increases the amount of detoxication, per gram of toxic substance. For instance, Waelsch & Weinberger (38) have shown that the amount of reduced glutathione in the blood stream is lessened after the ingestion of phenylacetic acid by human beings. They believe that this is due to the fact that the tripeptide is used up in the formation of glutamine.

On the contrary, Harrow, Mazur & Sherwin (36) did not find an increase in the acetylation of *p*-aminobenzoic acid when this substance was fed to rabbits after the simultaneous injection of glutathione.

## SITE OF DETOXICATION

The consensus of opinion seems to be that the seat of detoxication in the animal organism is the liver, while other tissues may or may not have the power to promote these reactions in a lesser degree. Thus Waelsch & Selye (39) report that partially hepatectomized mice are more susceptible to the toxic action of avertin than normal animals, owing to a decreased ability to detoxicate the drug.

Marenzi (40) extirpated the intestine, from the pyloric end of the stomach to the anus, in dogs. The animals lived from two to five days. Urinary phenols diminished progressively, indican disappeared in twenty-four hours, and conjugated phenols, in general, disappeared. The authors also showed that the ablation of the liver did not prevent conjugation. This would seem to indicate that the intestinal tract is the principal seat of phenolic conjugation.

Quick (41) fed benzoic acid and glycine to four nephrectomized dogs. Hippuric acid could not be found in appreciable amounts in the blood of dogs in which the kidneys were intact, but if the ureters were ligated, the feeding of 2 to 4 gm. of benzoic acid with glycine or gelatin produced 0.03 gm. of hippuric acid in the total blood. Injections into nephrectomized dogs of phenylacetic acid, however, led to the presence of phenaceturic acid in the blood, while injection of hippuric acid resulted in the appearance of free benzoic acid and benzoyl glucuronic acid. Feeding of benzoic acid and glycine to a nephrectomized rabbit produced hippuric acid in the blood, liver, and muscles. Quick concludes that the enzyme which synthesizes hippuric acid in the dog is limited to the kidney, while that which hydrolyzes hippuric acid and that which synthesizes phenaceturic acid are present elsewhere. There is apparently no localization of either enzyme in the kidney of the rabbit.

Boku & Kin (42) believe that urinary glucuronic acid serves as a reliable liver-function test. Eighteen patients with normal livers were chosen. Their urine contained, on the average, 0.013 per cent glucuronic acid when camphor had not been administered, while after the administration of 0.5 gm. of camphor these same patients showed 0.057 per cent of glucuronic acid in the urine. In fifty-five instances of "hepatic patients" practically no glucuronic acid was found in the urine, and after the administration of camphor, only traces of glucuronic acid could be shown. In catarrhal jaundice, urinary glucuronic acid was found to be proportional to the duration of the jaundice. Rabbits were poisoned with phosphorus and carbon tetrachloride causing liver damage. In these cases there was a decrease in the production of camphor glucuronic acid.

Kanzaki (43) believes that the liver must be concerned with synthesis of hippuric acid in the rabbit, since phosphorus and chloroform poisoning, which damage the liver, interfere with the formation of hippuric acid. After benzoic acid is injected into nephrectomized animals, hippuric acid appears. He also believes that the

spleen plays no rôle in this synthesis, for splenectomy and nephrectomy together give the same result as does nephrectomy alone. When the reticuloendothelial system of the nephrectomized rabbit is blocked with india ink and the animal is then injected with benzoate, hippuric acid shows a decrease, as compared with that of the non-blocked animals, in which increases are observed. This is probably due to the acceleration of cellular activity.

In their work on glucuronic acid, Hemingway, Pryde & Williams (27) sought to determine the site of conjugation of this substance. They devised a "pump-lung apparatus" with which they could perfuse various tissues with blood. They perfused first the kidney, then the kidney-liver preparation, next the kidney-spleen preparation, and lastly the kidney-limb preparation. They first proved that the isolated kidney could eliminate preformed glucuronic acid conjugates. After this, glucuronogenic drugs were added to the perfusate and various preparations tested. As has already been pointed out, it was found that the main if not the only site of glucuronic acid conjugation is the liver.

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## THE HORMONES\*

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*Oestrone and related hormones.*—Analysis of molecular refraction and hydrogenation of  $\alpha$ -oestrone and derivatives gives new evidence that an aromatic nucleus is present in their molecules. The water solubility of  $\alpha$ -oestrone is found to be 2.1 mg. per litre [Butenandt & Westphal (1)].

The work of Kofler & Hauschild affords a good basis to account for the discrepancies in the melting points of oestrone. They have found that oestrone can be obtained in three forms: a monoclinic metastable form melting at 256°, a rhombic metastable form melting at 254°, and a rhombic stable form melting at 259°.

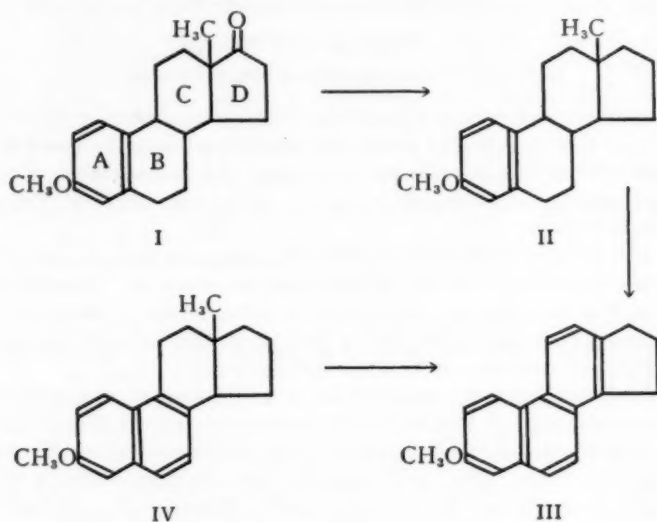
Cook & Girard, by reducing the ketone group of the methyl ester of oestrone (I), have obtained a methoxyoestratriene (II) which by dehydrogenation with selenium gives 7-methoxy-cyclo-1,2-pentenophenanthrene (III). The same product has been obtained by Cohen, Cook, Hewett & Girard by similar treatment of methylated desoxo-equilenine (IV), showing the identity of the basic structure and position of the hydroxyl group in the two hormones. Synthesis of compound III leaves no doubt in regard to its structure: it clearly shows that in the hormones, ring A is aromatic and that the hydroxyl group occupies the same position as it does in cholesterol.

From the preceding formulae it is clear that the methyl group is eliminated during the dehydrogenation. Butenandt & Thompson, on the other hand, isolated only chrysene by dehydrogenation of oestrone with zinc and selenium. The conclusion may be reached that the methyl carbon enlarges the pentatomic ring.

An important source of oestrogenic hormones has been found by Zondek in the urine of stallions. It is possible to obtain from 10,000 to 400,000 mouse units per litre of urine, and from 1,000 to 10,000 mouse units per kg. from the feces. Only small amounts are present in urine of either castrated or sexually immature horses. The testis

\* Received January 28, 1935.

seems to be responsible for the production of the oestrogenic hormone. Indeed it actually is the tissue where the female hormone can be found in the highest concentration; 350 gm. of testis yielded 23,100 units of oestrone.



The excretion of considerable amounts of oestrone has been observed in other Equidae. The male zebra eliminates 40,000 units per litre of urine and the male ass 4,000 mouse units.

Extracts of stallion urine had qualitatively the same physiologic activity as  $\alpha$ -oestrone (Zondek). Subsequently Häussler and also Deulofeu & Ferrari succeeded in obtaining from the stallion urine a substance responsible for the greater part of its oestrogenic activity and identified it as  $\alpha$ -oestrone.

Zondek explains the abundant production of oestrone by male animals by assuming that it is a transformation product of the male hormone which is produced in large amounts in the testis. Most of it would be transformed into oestrone by dehydrogenation, and then excreted. In females, the male hormone would also be the initial stage in the formation of the oestrogenic hormones. It would thus be possible to account for the constant presence of female hormone in urine of males, and also male hormone in the urine of females.

The existence of a  $\beta$ -oestrone of lower activity than  $\alpha$ -oestrone has been questioned by Jongh, Kober & Laqueur and also by Curtis *et al.* They have pointed out that the main difference, which is the property of  $\beta$ -oestrone to give a benzoate of double melting point, is also an attribute of  $\alpha$ -oestrone from various sources. The same property belongs to the oestrone from stallion urine and also to the international standard supplied by the Medical Research Council of Great Britain (Deulofeu & Ferrari).

Zondek & Euler have studied the excretion of oestrone by men and women. Both in the young and the aged, they found 5 to 30 mouse units per litre of urine, regardless of sex. With sexual maturity the amount increases in the urine of women to 50 units per litre during the post-menstrual period, 70 units during the intermenstrual stage, and 300 units at the moment of follicular growth. According to Eng, the urinary and fecal oestrone of men has its origin in the foodstuffs. Siebke estimates that the urinary and fecal excretion of oestrone amounts to 10,000 mouse units during a complete sexual cycle of the female; only by ingestion of 1,500 units a day does this amount increase. Osterreicher and Kemp & Pedersen-Bjergaard give similar figures and have found that only 6 per cent of the oestrone ingested or injected is excreted in the urine. Robson *et al.* could recover from urine no more than one-fifth of the amount injected; Zondek only about 4 per cent. This investigator, analysing the whole body of rats treated twenty-four to forty-eight hours previously with known amounts of oestrone, was able to detect only 20 and 6 per cent, respectively, of the amount injected. As the rest was not excreted, it must have been inactivated somewhere, probably in the liver, as he suggests. The benzoate of oestrone is not inactivated.

Most of the oestrone excreted during pregnancy seems to have an extra-ovarian origin. Brindeau, Hinglais & Hinglais (1) have confirmed the fact that variations from the normal cannot be found in the urine, blood, and placenta of a pregnant oophorectomized woman in so far as the content of oestrone is concerned. This would indicate its placental origin. The same results were attained by Hart & Cole on an experimentally ovariectomized pregnant mare. According to Brindeau *et al.* (2), urinary oestrone rapidly vanishes during the immediate postpartum period.

Whereas the placental origin for most of the oestrogenic hormones produced during pregnancy is not questioned, little is known

about the chemical nature of the first produced substance. Collip, Browne & Thomson have confirmed the isolation of trihydroxy-oestrin from placental extracts. They also found emmenin—a hydrolyzable compound containing trihydroxyoestrin, in the ether-soluble portion. As emmenin and trihydroxyoestrin are very active only on animals with ovaries, they probably need to be activated through ovarian influence.

Bialet-Laprida and Calatroni agree that the daily injection of high doses of oestrone to normal adult female rats produces a reduction in the weight of the ovaries. At the end of the month there is atrophy and involution; but if the administration is continued for a longer period (ninety days) the atrophic action disappears and the ovaries regain their normal weight, size, and microscopic aspect.

The antimasculine influence of oestrone has been confirmed [Korenchevsky & Dennison (1)]. It produces enlargement of the seminal vesicles of castrated males, an action which is probably not specific (David, Freud & de Jongh). Oestrone also potentiates the effects of the male hormone on the seminal vesicles [Korenchevsky & Dennison (2)].

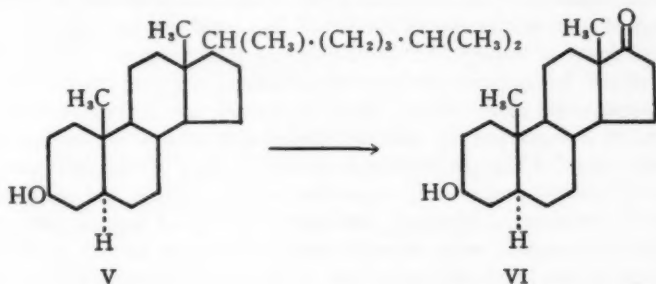
In agreement with the former work of Kaufmann, the ovary, according to Loeser, secretes from 250,000 to 300,000 mouse units of follicular hormone monthly, and from 40 to 50 rabbit units of luteal hormone. As these hormones are not excreted, they must be largely destroyed.

Hohlweg reports that high doses of oestrone induce the formation of corpora lutea in the ovary and a considerable enlargement of the hypophysis with abolition of its sexual action. Very high doses of oestrone may produce abortion in the doe (Courrier & Raynaud).

Many contradictory results have been reported concerning the influence of oestrogenic hormones on the growth and flowering of plants. Janot has found that equilenine, oestrone, equiline, and dihydro-oestrone hasten the growth of the hyacinth and *muguet*, but the attempts of Euler & Zondek, and Virtanen *et al.*, to repeat the experiments of Schoeller & Goebel (1) were negative as regards any growth-promoting action. The latter investigators (2), however, reaffirm the results of their former experiments.

*Male hormone.*—Ruzicka *et al.* have performed a partial synthesis of the male hormone transforming *epi*-dihydrocholesterol (V) into a substance identical with the male hormone (androsterone) isolated by Butenandt and Frattini & Maino from the urine of men. Ru-

zicka and associates proceeded to oxidize certain acetylated saturated sterols, and subsequently studied the resultant ketone substances with reasons for believing that one could be found having the structure suggested for the male hormone. From dihydrocholesterol acetate, they isolated compound VI, which, when administered in somewhat high doses, induced growth of the comb in capons (Ruzicka, Goldberg & Brünnger).



If *epi*-dihydrocholesterol, coprosterol, and *epi*-coprosterol acetates are similarly oxidized, ketones are obtained which are stereoisomers of compound VI. The one obtained from *epi*-dihydrocholesterol, which differs from VI in the inverted spatial position of the alcoholic group (m.p. 182°–183°;  $[\alpha]_D = -94.6^\circ$ ), is active as regards its comb-growth-promoting capacity even when doses as small as 70  $\gamma$  are injected daily during six days. Further test proved it to be identical with a sample of testicular hormone isolated from urine. The ketone obtained from dihydrocholesterol is active only when doses amounting to 500  $\gamma$  are used, and those of epimeric coprosterols were inactive even after injection of 1000  $\gamma$  a day, a fact affording new proof of the importance of the spatial structure as regards the physiologic action of the substance.

Whether this substance is the only existing male hormone is still unsettled; Gallagher & Koch have pointed out that, in contrast to the hormone obtained from urine, the one obtained from the testis of the bull is easily destroyed when boiled with alkali.

*Corpus luteum*.—The active principle responsible for the progestational modifications of the uterus (progestin) has been isolated in four different laboratories. Two active substances have been found, one melting at 128 to 129°, of formula  $\text{C}_{21}\text{H}_{30}\text{O}_2$ , called by

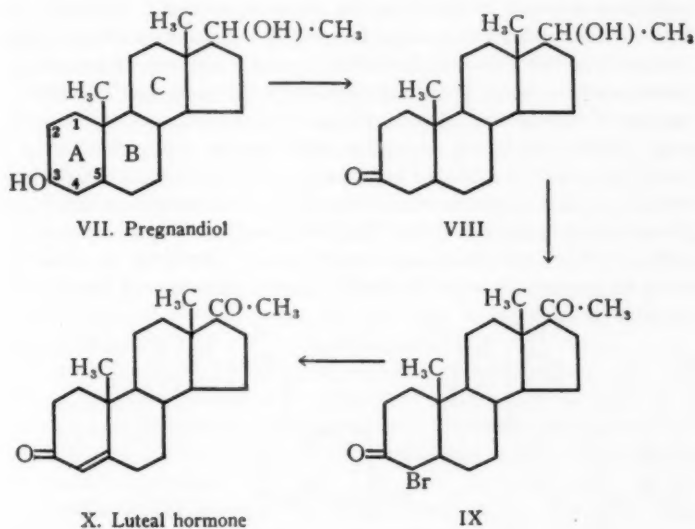
Slotta, Ruschig & Fels "luteosterone C," and the other, "luteosterone D," having the same constitution but melting at 120 to 121° [Slotta, Ruschig & Fels (1); Butenandt, Westphal & Hohlweg; Hartmann & Weltstein; and Wintersteiner & Allen]. Although there is general agreement as to the chemical and physical properties of both substances, there remain some discrepancies as to whether they are equally active. Fels, Slotta & Ruschig find that administration of luteosterone C, in doses amounting to 1 mg., produces only a condition of uterine congestion, similar to that produced by injection of high doses of oestrone, but quite different from progestational modifications. According to the same investigators, it is possible to induce progestational modifications, when the Corner test is used, with 1.2 mg. of luteosterone D, although perceptible effects are noticeable with even 0.4 mg. Its hyperemic action is weak. If female rabbits are previously prepared by administration of oestrone, the progestational condition is obtained with only 0.9 mg. of luteosterone D. The C compound, when injected with luteosterone D, has a potentiating action, with the result that, if mixtures are used (60 or 70 per cent C + 30 or 40 per cent D), a positive Corner test can be obtained using 0.5 mg.; there is congestion and a progestational condition. If the Clauberg test is used only 0.12 mg. of the mixture is required, whereby the inference is drawn that 1 Corner unit is the equivalent, when the mixture is used, of 4 Clauberg units.

Butenandt, Westphal & Hohlweg, using a modified Clauberg technique, have found, however, that the substance melting at 128.5° induces a condition of pseudo-pregnancy in female rabbits when injected in doses of 0.75 mg. Wintersteiner & Allen also tried the activity of the two substances and found that both produce the same progestational effect; they estimate the Corner unit to be approximately 1 mg.

In addition to the active substances present in corpus luteum, all the investigators find a physiologically inactive product,  $C_{21}H_{36}O_2$ , melting at 194°. Slotta, Ruschig & Fels call it "luteosterone A" and say that it is accompanied by another inactive substance, "luteosterone B," which they do not describe further.

Earlier research indicated that the active substances melting at 128 to 129° and at 120 to 121° were diketones with a double bond. Butenandt, Westphal & Cobler, on the basis of a probable relationship between these hormones, pregnandiol, and the follicular and testicular hormones, suggested formula X as a probable one for the luteal hor-

mone (m.p. 128°). Simultaneously, Slotta, Ruschig & Fels arrived at a similar formula, partly on the basis of crystallographic studies of Neuhaus. An explanation was not given to account for the difference between luteosterones C and D. The spectroscopic studies of Wintersteiner & Allen indicated an  $\alpha : \beta$  position for the double bond, and also in both luteosterones C and D, an absorption band at 240  $\mu$ , characteristic for the above-mentioned ketones (Menschick, Page & Bosser). This was subsequently confirmed by Slotta, Ruschig & Blanke.

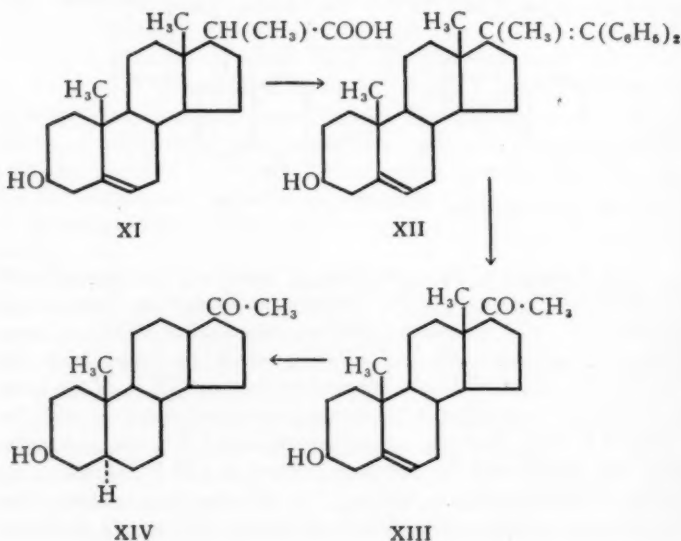


A confirmation of the correctness of formula X has been afforded by the partial synthesis of the hormone performed by Butenandt & Schmidt (1). By adequate oxidation, pregnandiol (VII) is transformed to pregnanol-20-one-3 (VIII), which, by bromination and subsequent oxidation, gives a monobromodiketone (IX). If the latter is treated with pyridine, a substance is obtained identical with the hormone isolated from the corpus luteum, m.p. 121°; but, if its solutions are seeded with the hormone melting at 128.5°, crystals with the same melting point are formed. The inference may be drawn that the different melting points of both substances are due to a dissimilar



crystalline form, rather than to a chemical isomerism. On the other hand, it is impossible to reconcile a double bond including the tertiary carbon 5, with a cis-trans isomerism between rings A and B, as formerly considered by Slotta, Ruschig & Fels (2) when they found that, by simple melting, the B substance can be transformed into C. The matter cannot be considered as definitely settled, and further research is required to determine the causes of the difference.

Another partial synthesis has been performed by Butenandt, Westphal & Cobler starting from oxy-bisnor-cholenic acid (XI), a substance obtained by Fernholz (1) from stigmasterol. Transformed into its methylated ester and subsequently treated with phenyl magnesium bromide, this acid produces a tertiary carbinol. Vacuum distillation of the latter gives a doubly unsaturated alcohol (XII), the acetate of which, treated with bromine, takes up a molecule on the ring double bond. By oxidation with ozone, benzophenone and acetylated dibromoxyketone are obtained. By subsequent elimination of bromine, and saponification, the acetyl product is transformed into the oxyketone proper (XIII). This substance, if oxidized by chromic acid, yields a mixture of compounds among which the hormone X must be present, because Fernholz (2) by oxidation of the bromi-



nated oxyketone XIII by means of permanganate, and subsequent elimination of bromine, has succeeded in obtaining the active product melting at 128 to 129°.

Concerning the inactive substance melting at 194°, Butenandt & Mamoli have shown it to be identical with pregnanol-3-one-20 (XIV) and, like the male hormone, to belong to the steric type of dihydro-cholesterol. Pregnandiol, found in the urine of pregnant women, may also be considered as a reduction product of the luteal hormones though it belongs to the coprosterol series.

Although the only hormone isolated from the corpus luteum is the one producing the condition of pseudo-pregnancy, Brouha & Desclin, from an evaluation of the activity of aqueous and alcoholic extracts of the gland in relation to the modifications of the pubic joint and mucification in guinea pigs, reach the conclusion that there probably is more than one active substance produced by the corpus luteum.

*Insulin.*—Fisher & Scott have determined the insulin content of the pancreases of cattle of different ages and found the following amounts in units per gram of tissue: in fetuses, up to five months, 33.2; between five and seven months, 23.1; in calves, from six to eight weeks, 11.4; two years old, 4.8; and in fully-grown animals, up to 1.8 units.

Scott found that crystalline insulin always contains zinc and showed that only when that metal or else cobalt, nickel, or cadmium is present, insulin crystals may be obtained. This explains why Bruch could obtain crystalline insulin only from some commercial samples, and failed with others.

Bürger & Kohl showed that crystalline insulin is less active than impure insulin when it is injected intraperitoneally, but the reverse happens if it is injected intravenously. Fisher & Scott (1), studying the peptic digestion of insulin, found a decrease in activity running parallel with a diminution in the tyrosine content of the molecule, while cystine remained unchanged and lysine increased. The possibility of there being several chemical groups of different biological activity in the insulin molecule led Davis, Luck & Miller to observe that if insulin is treated by X-rays or by acid-alcohol, the blood-sugar-lowering capacity vanishes more quickly than the blood-phosphorus-decreasing power.

The fact is now soundly established that the nervous system is not essential for an almost perfectly regulated insulin secretion (Gayet

*et al.*, Houssay *et al.*, and Meythaler *et al.*). Ablation of the pancreas in a dog progressively produces: (a) rapid decrease of hepatic glycogen; (b) decreased glycogen-fixing capacity of the muscle after injection of glucose; (c) slower recovery of the muscle glycogen after fatigue; (d) a lower glycogen content of the muscle even at rest (Foglia & Fernández; Dambrosi). All these disturbances are compensated for, and sometimes even overcompensated, by administration of insulin. As section of both vagi of either cats or dogs does not bring about such disturbances, the conclusion must be drawn that secretion of insulin continues normally (Foglia & Fernández; Dambrosi). The nervous system, however, plays an auxiliary rôle, contributing to a quicker and finer adjustment of insulin secretion. Thus, when the vagi are cut, there is a slower recovery of the blood-sugar level following injection of either glucose or insulin (Etcheverry, unpublished data).

Insulin secretion increases during the post-absorption sugar flood, as shown by blood transfusion in man (Boller, Überraek & Falta). According to London & Kotschneff, this hypersecretion is partially conditioned by the vagus nerve.

The injection of glycine produces a condition of hyperinsulinemia consecutive to a primary discharge of epinephrine. The hypoaminoacidemia produced in normal animals by injection of insulin is due entirely to an increased secretion of epinephrine (Luck & Morse, Davis & Van Winkle). The observations of Lacquet, de Nayer & Bouckaert and of Bouckaert *et al.*, that insulin hastens the elimination of simultaneously injected glycine, might thus be explained by the hyperadrenalinemia produced.

*Anterior pituitary.*—Much work is now in progress on the anterior lobe of the hypophysis. It all shows that its position within the endocrine system is a central one, and also how greatly growth and metabolism depend on its function.

The anterior lobe of the pituitary is essential for the anatomic integrity and normal function of the thyroid. The atrophy of the vesicular epithelium following hypophysectomy has been consistently confirmed in rats (Loeser & Thompson), fowls (Towner-Hill & Parkes), guinea pigs (Macchiarulo & Amelotti), toads (Magdalena), etc. After hypophysectomy the compensatory hypertrophy consecutive to a partial removal of the thyroid, is altogether lacking (Kahler, Magdalena). Besides the already well-known signs of hypothyroidism, hypophysectomized dogs show an increase in plasma globulin (I. Gold-

berg) and decreased phagocytic activity of leucocytes (Parodi). The basal metabolic rate is about 26 per cent below the normal in hypophysectomized rats (Pugsley, Anderson & Collip) and about 16 per cent below in dogs (Houssay). Sturm (confirming Houssay, Biasotti & Mazzocco) found an increase, followed after some weeks by a decrease, in the blood-iodine content of hypophysectomized animals.

According to Houssay the condition of hypophysectomized dogs is only one of hypothyroidism, rather than one of athyroidism, because after thyroidectomy the basal metabolic rate falls still further. Hypophysectomy, on the other hand, does not decrease the metabolism of thyroidectomized animals. Anterior-lobe extracts increase the metabolic rate (+60, +128 per cent in dogs) if the thyroid is present; the influence of the pituitary on basal metabolism is exerted through its action on the thyroid. The specific dynamic action was normal in twenty hypophysectomized dogs, but it decreased when the thyroid was removed also.

Purification of the thyreotropic hormone by Collip (1) allowed its separation from the gonad-stimulating and adrenotropic principles, giving a highly active product. On the other hand, peptic digestion or autolysis of the anterior lobe destroys the gonad-stimulating factor before the thyreotropic factor (Guyénot, Ponse, Vallette & Dottrens).

The fact that the thyreotropic hormone increases the metabolism of rats, dogs, guinea pigs, fishes, and men, has been confirmed (Pugsley, Anderson & Collip; Houssay; Schoedel; Black; Wachstein). In the absence of the thyroid, however, it decreases the metabolic rate (Schoedel).

The sensitivity of the thyroid of a given species against the thyreotropic hormone is inversely proportional to the amount of hormone produced by its own hypophysis (Collip, confirming Aron). Pieper and Eitel confirmed the finding that the hormone acts even on denervated thyroids. It is interesting to point out that ascorbic acid, although a different substance, also has a thyreotropic action (Heyl).

Rats (Pugsley & Anderson) and men (Barr) show creatinuria when treated with the thyreotropic factor. The excretion of calcium increases and the balance becomes negative for a while (Pugsley & Anderson). Hepatic glycogen decreases (Eitel & Loeser; Silberstein, Gottdenker & Geiger) and vitamin A entirely disappears (Schneider & Widmann); sodium decreases in the plasma and increases in the

skin (Eggs); post-absorption lipemia is either decreased or abolished (Silberstein, Gottdenker & Geiger).

Following thyroidectomy, the basophilic cells increase in the anterior lobe of the pituitary; the cells appear similar to those produced by castration, and the eosinophilic ones decrease in number (Severinghaus, Smelser & Clark). In the rat there is also a slight but clear diminution of thyreotropic and gonadotropic activity (Reforzo, unpublished). Castration augments the thyreotropic action of the hypophysis (Loeser). Small doses of iodine inhibit, and high doses augment the thyreotropic action. The effect of iodine on the thyroid seems to be exerted through the mediation of the hypophysis (Loeser; Loeser & Thompson).

According to Collip, confirmed by Hertz & Kranes, if the administration of thyreotropic hormone is prolonged during two to three weeks, the basal metabolism falls below normal and the vesicular epithelium of the thyroid grows flat, becoming inactive. The blood serum and the organs of animals so treated may now neutralize several doses of thyreotropic hormone, but not thyroxin. The anti-thyreotropic substance may be highly concentrated, is thermolabile ( $100^{\circ}$  at pH 3), and its origin is unknown (Anderson & Collip; Collip). Loeb regards the annihilation of the thyreotropic action as a local phenomenon occurring in the thyroid itself.

The pituitary body seems to exert a hormonal influence on the parathyroids. In 66 per cent of hypophysectomized dogs Houssay & Sammartino have found atrophic and degenerative lesions of the parathyroids; the lesions were constant and severe in dogs deprived of both hypophysis and pancreas (confirmed by Collip). The calcium content of the blood, however, remains normal in hypophysectomized dogs (Marenzi & Gerschman) and rats (Collip), although it falls to 7 to 9 mg. per cent in the plasma of hypophysectomized and depancreatized dogs (Marenzi & Gerschman, unpublished data). The calcium balance of hypophysectomized rats tends to be negative, but the growth-promoting extract renders it positive (Pugsley). The hormone of the parathyroids raises the blood-calcium concentration of hypophysectomized rats (Pugsley), but their ability to form new bone remains below normal (Collip). Osteoporotic lesions are frequent in cases of human pituitary basophilism; Cushing found enlarged parathyroid glands in four of six patients.

According to Anselmino, Hoffmann & Herold, the extract of the anterior lobe of the hypophysis has a parathyreotropic action which

may produce parathyroid hypertrophy with increase of clear cells, decrease of dark cells, and disappearance of the oxyphilic cells. The same effect is evoked by the alcoholic precipitate of pregnancy urine which does not exert a thyreotropic influence.

The parathyreotropic factor has not been obtained clearly separated from other hormones, but it does not pass by ultrafiltration through collodion membranes. Its administration to dogs induces a prolonged increase in the blood-calcium content [Hoffmann & Anselmino, confirmed by Marenzi & Gerschman (unpublished data)]. Hertz & Kranes have also observed hypertrophic and hyperplastic reactions in the parathyroid glands of rabbits treated with either anterior-lobe extracts or pregnancy urine.

The adrenotropic (or corticotropic) factor has been isolated by Anderson & Collip (and Collip). After separation of the thyreotropic factor by precipitation with ammonium sulphate, dialysis, and precipitation with alcohol, a product is obtained which hastens the compensatory hypertrophy of the remaining adrenal when one has been ablated. It also compensates for the adrenal disorders consecutive to the ablation of the hypophysis, but does not improve the cachexia (Collip). Anselmino, Hoffmann & Herold report the isolation of the corticotropic principle from all the others. It is found with the pancreatropic hormone in the ultrafiltrate obtained at pH 5.3, but the latter can be destroyed by a short period of boiling. When injected into young castrated rats, this substance induces a marked hypertrophy of fasciculated and glomerular layers of the adrenal cortex.

Prolonged administration of certain anterior-pituitary-lobe extracts may lead to low glycemic levels (E. Evans; Shpiner & Soskin). According to Anselmino, Herold & Hoffmann, the anterior lobe of the hypophysis contains a pancreatropic factor because its repeated daily injection into rats produces hypertrophy of the islands of Langerhans. It is possible to separate it from the gonad-stimulating, growth-promoting, thyreotropic, and ketogenic factors, because it passes on ultrafiltration through collodion membranes at pH 5.3; it goes through with the adrenotropic factor, but, by boiling, the latter is destroyed. The injection of the pancreatropic factor produces a fall in blood sugar in dogs, and lessens the rise in blood sugar provoked by the administration of glucose. The pancreas is necessary for these effects to appear.

The plurality of gonad-stimulating substances of the hypophysis, blood serum, urine, and certain tissues, has continued to be confirmed.

A gonad-stimulating substance acting on the ovary has been found in onions (Peissachowitsch) and a similar one in alfalfa (Friedman & Friedman).

It is well known that the gonad-stimulating factor of pregnancy urine is different from the hypophyseal one: (a) it does not produce an illimitable ovarian growth; (b) it is inactive in birds; (c) its activity is feeble in hypophysectomized rats; (d) it has no influence on the macaque [it is active, however, on the Algerian female monkey (Courrier & Gros)]; (e) the relative strength on rats and mice is different. Pregnancy urine produces hypertrophy of the interstitial cells of the ovary when injected into hypophysectomized rats; there is also partial luteinization of thecal cells and secretion of oestrone, but neither follicular ripening nor large corpora lutea can be seen (Smith & Leonard). Strikingly different are its effects on the normal sexually immature rat where it produces follicular ripening, abundant formation of corpora lutea, and increased weight, as if the injected urine excited the hypophysis to secrete a synergistic or activating factor. A principle of this kind must be present also in the urine after the menopause and after oophorectomy (Leonard & Smith, Anselmino & Hoffmann), in fresh anterior-pituitary extracts in 40° alcohol, and in the urine of patients with testicular tumors, since the injection of any of them into hypophysectomized rats, together with pregnancy urine, develops a marked ovarian hypertrophy, follicular ripening, and intense luteinization.

After the menopause and after oophorectomy the urine contains a gonad-stimulating factor supposedly originating in the anterior hypophyseal lobe on account of its similarity in action to the hypophyseal principle. If they are injected into sexually immature hypophysectomized rats, they both produce follicular ripening, proliferation of cells of the granulosa, and, occasionally, partial luteinization (Leonard & Smith); in guinea pigs (Leonard) and in very young rats (Hamburger) follicular ripening is produced, and also oestrus in the macaque (Smith & Engle); in young chickens they have a stimulating action on the testis and on the growth of the comb (Hamburger). Pregnancy urine has none of these actions.

A gonad-stimulating factor similar to the hypophyseal one is also present in the blood serum of the pregnant mare; although it has very similar properties to the hypophyseal factor, the findings concerning its action are better explained by assuming it to be placental in origin (Catchpole & Lyons).



Certain synergistic factors produce intense gonad-stimulating action when injected at the same time as pregnancy urine, inactive by itself: anterior pituitary lobe allows a marked luteinizing action on monkeys (Engle); post-menopause urine (Leonard & Smith), and alcoholic extract (40°) of sheep hypophysis, on hypophysectomized rats (Evans, Pencharz & Simpson). The hypophysis of rats less than twenty-one days old does not exert a gonad-stimulating action when implanted in hypophysectomized rats, although it contains the synergistic factor because it is active in normal rats older than twenty-one days (Swezy).

According to the important experiments of Smith, Engle & Tyndale and Evans, Pencharz & Simpson there seem to be two different gonad-stimulating factors acting on the testis: one gametogenic, influencing only the germinal cells, and the other acting preferentially on the interstitial tissue, thereby governing the production of male hormone. Good support is thus afforded for Ancel & Bouin's theory regarding the interstitial cells as the site of production for the male hormone.

Fevold & Hisaw believe the synergistic factor to be the same as the follicle-stimulating hormone, but unfortunately they have not experimented on hypophysectomized rats. According to Lipschütz there are three gonad-stimulating factors: an oestrogenic one, another sensitizing the follicle to be luteinized, and finally, a luteinizing one. Post-menopause urine contains the sensitizing factor (produces oestrus but no luteinization); the hypophysis of both the female rat and guinea pig contains the oestrogenic and luteinizing factors (produces oestrus in the rat and corpora lutea in the doe) but lacks the sensitizing one. If a hypophyseal extract and the urine are injected together, a marked luteinization occurs also in rats. Brindeau, Hinglais & Hinglais also admit the existence of three substances.

The preparation of two hypophyseal substances in almost pure form, the one producing follicular maturation, and the other luteinizing, has been reported by Fevold & Hisaw and Wallen-Lawrence. Experiments of Smith & Engle lead them to accept the existence of two substances. Methods have also been reported for isolation of the hypophyseal (Collip) and urinary (Katzman & Doisy and Davy) gonad-stimulating substances. Ovulation of the doe has been studied as an assay method by Towner-Hill & Parkes. Just as for enzymes or proteins, heat (62°), hydrogen peroxide, or ultraviolet rays destroy the gonad-stimulating substances of pregnancy urine (Euler

& Zondek). Collip, however, has obtained from the anterior lobe of the pituitary a follicle-maturing substance which is not destroyed during one hour of boiling.

Dumont, D'Amour & Gustavson have confirmed the possibility of inducing ovulation in the doe by transfusion of large amounts of blood from another recently impregnated doe. Neither the existence of the ovum nor the aspiration of the follicular contents produces luteinization unless prolactin is present (Zondek). Ovulation and spermatogenic discharge have been confirmed in fishes injected with anterior-pituitary-lobe extracts (Pereira & Cardoso). Pregnancy urine induces ovulation in the amphibians *Xenopus* and *Rana* (Bellerby); its inactivity in the toad is well known.

The existence in the hypophysis of substances inhibiting the gonad-stimulating factor has been confirmed (Collip; Leonard). Both pregnancy urine and anterior-pituitary-lobe extracts may produce hypertrophy of the clitoris in guinea pigs (Papanicolaou & Falk; Guyénot, Ponsé & Trollet).

The interesting fact has been observed that after prolonged administration of gonad-stimulating substances (pregnancy urine and anterior pituitary lobe) their effects on the ovary grow weaker and the organ regains its normal size or even becomes smaller. A resistance toward both substances has been developed (Selye, Collip & Thomson; Selye, Bachman, Thomson & Collip).

Depending on the species, hypophysectomy has different effects on pregnancy. If performed during the first half of pregnancy, fetal resorption ensues in rats and mice; if done later on, gestation is prolonged in the rat; in mice, abortion does not occur (Selye, Collip & Thomson). In guinea pigs, if operated on after thirty-four to thirty-six days, there is fetal resorption within two days; if hypophysectomy is performed after forty or forty-one days, neither gestation nor parturition is affected (Pencharz & Lyons). In the bitch abortion invariably follows two or three days after hypophysectomy (Houssay, unpublished results confirming the work of Aschner).

The lactogenic action of certain anterior-pituitary-lobe extracts has been confirmed by many investigators. With Sardi we have confirmed the fact that in order to obtain milk secretion a fully developed mammary gland is necessary, and also that the lactation-inducing factor may act even in males (guinea pigs and dogs) previously treated with oestrone in order to produce the necessary mammary growth (Sardi; Houssay, unpublished data). Hypophyseal extracts

also produce crop-milk secretion (Fremery & Spanhoff, confirming Riddle). The methods of assay have been studied by Nelson.

"Prolactin" seems to be a protein (Bates, Riddle & Lahr). It is different from either the growth, the maturity, or the thyreotropic fractions (Collip, confirming Riddle). Its lactation-inducing capacity is independent of the hypophysis, the ovary, the thyroid, or the adrenal glands; it persists even after simultaneous removal of the three first-mentioned glands (Houssay).

Hypophysectomy abolishes the milk secretion of rats, mice (Collip *et al.*), and dogs (Houssay). If hypophysectomy is performed on rats during the second half of pregnancy, the mammary glands continue to grow, and after parturition, there is milk secretion for a few hours (Collip considers this to be due to a possible uterine substance). In the bitch abortion follows after one to three days and lactation does not ensue; only a few drops of a clear liquid are produced. Posterior lobe removal or injuries of the tuber cinereum usually do not interfere with lactation (Houssay). Caesarean section produces lactation in the rat only when the hypophysis is present (Collip). In guinea pigs it is necessary to ablate the ovaries in order to obtain lactation after removal of the uterine contents (Nelson). The ovarian hormone promotes mammary gland development and inhibits milk secretion. After parturition, the ovarian influence diminishes and the hypophyseal one predominates as regards lactation. If, by injection of placental extracts, a condition of hyperovarism is induced in rats, and the ovaries are then removed, milk secretion ensues provided the hypophysis is present (Nelson).

It is well known that in many species the secretory activity lasts as long as the animals are suckled or milked regularly. According to Selye this is due to a nervous reflex initiated by suckling; the lactogenic factor is secreted and distributed alike to all mammary glands. Suckling inhibits the sexual cycles and maintains large corpora lutea. In female rats a condition of pseudo-pregnancy with mammary development and abolition of sexual cycles may be produced by suckling (Selye & McKeown). Suspension of menstruation during lactation in women might be explained in a similar way.

Early involution of the thymus has been again described by Houssay & Lascano-Gonzalez in sixteen of twenty hypophysectomized puppies.

Parodi has found a mild anemia with eosinophilia and increased resistance of the red cells. The activity of leucocytes as regards

phagocytosis is very low, probably on account of the decreased thyroid function (Parodi). The spleen of hypophysectomized dogs shows larger follicles and a greater number of them than normal animals (Houssay & Lascano-Gonzalez).

Collip has continued his attempts to obtain a highly purified growth-promoting hormone. To maintain its activity, it must be preserved from exposure to air and should be kept very cold; alkali protects it (Rubinstein). Rats hypophysectomized when very young grow to reach about 60 gm.; if heavier rats are deprived of their hypophyses, growth immediately stops. A hypophysectomized rat weighing about 100 gm. is the most suitable test object for determining the activity of the growth-promoting hormone (Collip). Simultaneous administration of thyroid augments the effect of pituitary growth-promoting extracts in man (Engelbach & Schaefer, confirming Smith in rats).

A ketogenic principle (for which the name *Fettstoffwechselhormone* has been suggested) is still present in ultrafiltrates that have lost their thyreotropic action. If the extracts are ultrafiltered at pH 5.3, the ketogenic factor does not go through and the ultrafiltrate exerts a glycogenolytic action when injected into rats (Anselmino & Hoffmann). Substances have been found in urine which increase the acetone-body and glucose concentrations of the blood (Harrow, Naiman, Chamelin & Mazur); they were isolated by adsorption on benzoic acid and shown to be different from the pituitary factor (Funk). The factor producing a decrease in the fat concentration of blood may increase the acetone-body content of blood a little (Steppouhn *et al.*; Leiner; Raab) and the thyreotropic, still more (Silberstein & Gottdenker). The anterior-pituitary-lobe extract increases ketonuria in rats (Butts *et al.*; Black, Collip & Thomson) even after thyroidectomy (Black *et al.*); this action is prevented by glucose (Deuel). Diabetogenic extracts of anterior pituitary lobe increase the acetone-body concentration of urine in normal dogs; the action is stronger after pancreatectomy; it persists when the gonads, splanchnic nerves, or adrenal medulla are removed, and when the tuber cinereum is injured, but it disappears, or is greatly reduced, when the thyroid is ablated (Rietti).

Raab reports the presence in both pituitary lobes of a substance which is able to lower for hours the blood-fat concentration when injected into dogs; he calls it "lipoitrin" and believes that its action is mediated by the tuber cinereum.

The anterior lobe of the hypophysis has a regulating action on

carbohydrate metabolism.<sup>1</sup> It promotes sugar production, and extracts of the gland, when given in high doses, inhibit glucose utilization; a diabetic condition is produced. The tendency of hypophysectomized animals to fall into hypoglycemia (Mahoney; Kepinow; Phillips & Robb) and their sensitivity to insulin (Barnes, Dix & Rogoff; Scott *et al.*) have been confirmed. Venous blood of hypophysectomized dogs does not produce hypoglycemia in the rabbit (Di Benedetto), but the venous blood coming from the pancreas of such dogs would, according to Kepinow & Guillaumie, exert a stronger hypoglycemic action than the pancreatic blood of normal animals. There seems to be an antagonism between insulin and the hormone of the anterior hypophysis, as shown by the fact that the blood of dogs with pancreatic diabetes contains a hyperglycemic substance which disappears if the hypophysis is removed (Kepinow). Biasotti, injecting glucose into ten hypophysectomized dogs, contrary to the findings of Kepinow, consistently observed, for each individual case, a slower recovery of the blood-sugar level as compared with ten normal dogs.

Muscle and hepatic glycogen decrease in hypophysectomized toads (Houssay *et al.*) and rats (Phillips & Robb) but remain unchanged in dogs.<sup>2</sup> Glycogenolysis is decreased in the liver of the hypophysectomized frog (Fluch, Greiner & Loewi). The loss of glycogen in rats and toads goes hand in hand with progressive asthenia and hypoglycemia (Houssay). In the toad there is also a decrease in heart glycogen, with bradycardia and increased chronaxie (Orias), and decreased concentrations of phosphagen, glutathione, and glycogen in the skeletal muscle, together with a lowered capacity to form lactic acid (Marenzi). Adequate implantations of anterior lobe, and partially also of posterior lobe, completely correct all these disturbances.

The favorable influence of hypophysectomy on the course of pancreatic diabetes of dogs has also been confirmed (Barnes *et al.*; Collip *et al.*; Soskin *et al.*; Kepinow; Davis; Lucke). Injuries of the tuber cinereum produce a similar effect (Davis). The hypophysectomized dog, deprived of its pancreas, shows glucose-tolerance curves ranging from normal to those found in pancreatic diabetes, most of them being intermediate (Biasotti).

Anterior-lobe extracts apparently produce the blood-sugar increase by two different mechanisms, as if there were two active

<sup>1</sup> Cf. also this volume, p. 186. (EDITOR.)

<sup>2</sup> The disturbances of carbohydrate metabolism following hypophysectomy are more marked in the toad, somewhat less in the rat, and even less in the dog.

factors. One produces a rapid, brief, and rather low (a few decigrams) blood-sugar rise (Lucke; Shpiner & Soskin; Holden & Raymond, etc.). The second acts in normal dogs following repeated daily injections, the hyperglycemia appearing after two or three days and reaching a high stable level (0.2 to 0.35 gm. per 100 cc.) if the extract is sufficiently active (preparation and conservation of the extract at a very low temperature is essential). The blood-sugar increase, thus obtained, shows four main characteristics: (a) it begins gradually; (b) it disappears or becomes negligible during fasting; (c) there is a typical glycogen increase; (d) if the injections are discontinued, it vanishes after two or three days. If the animals are fed on a high-carbohydrate diet, the blood-sugar rise is easier to obtain than under the usual meat diet. The presence of the thyroid, the adrenal medulla,<sup>3</sup> the gonads, or the hypophysis is not essential for the development of this hypophyseal diabetes with glycosuria, ketonuria (absent or negligible in thyroidectomized animals), and increased lactic acid content of the blood. If glucose is injected under these circumstances a reduced utilization of sugar may be observed: there is a prolonged blood-sugar rise and little or no increase of the respiratory quotient (Biasotti). The properties of the diabetogenic substance have been studied by Houssay, Biasotti & Rietti.

According to Anselmino & Hoffmann the hypophysis produces a glycogenolytic factor, different from all the others. Following ingestion of a sugar-rich diet there appears in the blood serum the same glycogenolytic principle (Anselmino, Hoffmann & Herold).

Protracted administration of either anterior-pituitary-lobe extracts or pregnancy urine brings about degenerative changes in the anterior lobe of the hypophysis (Collip, Selye & Thomson). Basophilic cells are particularly affected by pregnancy urine (Wolfe; Severinghaus).

*Posterior pituitary.*—Dietel, by extraction with barium hydroxide, obtained a highly purified and active melanophore-expanding hormone which produces capillary dilatation and fall in blood pressure. Orias has shown the great physiological importance of the posterior lobe in maintaining a normal blood pressure in the toad; after total hypophysectomy the blood pressure markedly falls from about 40 mm. of mercury to 27 mm. at the end of the week and to about 17 mm. at the end of the month. Ablation of the glandular lobe

<sup>3</sup> This is opposed to the suggestions of Barnes *et al.*, Anselmino & Hoffmann, and Lucke, who ascribe the hyperglycemic action of the hypophysis to its influence on the adrenal medulla.

produces a slight fall only after some weeks, when general asthenia is well developed. Following injury of the tuber cinereum the blood pressure remains unchanged. Aubrun & Porto found dilatation of the skin capillaries of the toad following total hypophysectomy; ablation of the anterior lobe alone did not produce it until general asthenia was present. Both the hypotension and the capillary dilatation may be compensated for by injection of extracts of any of the hypophyseal lobes, the posterior one being more active. The blood pressure of hypophysectomized dogs is somewhat lower (10 to 30 mm. of mercury) than in normal dogs and the recovery after bleeding is retarded (Braun-Menendez). The blood pressure is also below normal in hypophysectomized rats (Wyman & Tum Suden).

Whether there is hypersecretion of the posterior lobe in eclampsia is still unsettled.

Marenzi confirmed the observation that posterior-pituitary-lobe extracts increase the lactic acid concentration of blood.

*Thyroid.*—Canzanelli, Harington & Randall have definitely proven that thyroxine is a tyrosine derivative; from *l*-tyrosine they obtained optically active thyronine, similar to the one obtained from *l*-thyroxine. Foster has shown that di-iodotyrosine, just as thyroxine, may restore the thyroxine content of the thyroid.

According to Lerman & Salter, the action of the thyroid on basal metabolism and myxedema depends more on the total iodine than on the thyroxine iodine; all the iodine present in thyroglobulin would, therefore, be calorigenically active. The thyroid substance should be assayed in terms of total organic iodine rather than thyroxine iodine. Di-iodotyrosine iodine is calorigenically active so long as it is part of the thyroglobulin molecule, but loses its activity when separated from the thyroxine-iodine fraction. The thyroxine polypeptide of Harington & Salter has the same effect as crystalline thyroxine when administered in equivalent dosage. According to Cavett, Rice & McClendon, the iodine content of thyroglobulin from persons with hyperthyroidism decreases as the basal metabolism increases.

The tissues of rats injected with thyroxine show an increased oxygen consumption.

Euler determines the thyroxine content of blood by the rate of decolorization of methylene blue in the presence of frog muscle; normally there is about  $10^{-18}$  gm. per cc., the amount increasing in hyperthyroidism (Euler & Holmquist).

Repeated injection of thyroxine increases blood fibrinogen and



hastens blood clotting in the rabbit (Zunz, Sanchez de la Cuesta & Vesselovsky). Thyroidectomy markedly increases the globulin content and viscosity of the blood and, somewhat less, the alkaline reserve and the non-protein nitrogen, whereas hypothyroidism decreases the globulin content and viscosity of the blood (Rossignoli, Di Benedetto, Guerrero & Di Benedetto). Thyroxin increases the fat content of blood and muscle and decreases that of the liver (Schmidt & Bradford); in the cow there is a marked increase of the milk fat (Graham). Thyroid administration to rats increases the calcium excretion, the balance becoming negative, but blood-calcium concentration remains unchanged (Pugsley & Anderson). Goiter has been produced in rats fed with yellow corn, oats, and distilled water; addition of calcium increased the goitrogenous capacity (Hellwig). Prolonged administration of oestrone produces atrophy of the vesicular epithelium (Bialet-Laprida; Karp & Kostkiewicz). Total thyroidectomy in a diabetic patient increased the sugar tolerance but at the expense of such troubles that no justification is given for such a treatment (Wilder *et al.*).

*Parathyroids.*—Tweedy, Bell & Vicens-Ríos, by gradual deamination of parathormone with isoamyl nitrite in concentrated acetic acid, observed a marked decrease of its activity when the amino nitrogen loss amounted to 35 to 40 per cent.

If parathormone is administered during a long period, its effects grow weaker and finally disappear. This fact prevents its prolonged therapeutic use (Thomson & Collip). There is a tendency to believe that hyperparathyroidism might be due to an influence of the hypophysis on the parathyroids (Albright *et al.*)

*Adrenal cortex.*—Kendall *et al.* report that they have isolated cortin in crystalline form. Its empirical formula is  $C_{20}H_{30}O_5$ . A structural formula has been assigned, but no further details are given. Cortin prevents the increased catabolism of proteins induced by thyroxin in either normal or adrenalectomized dogs and may even bring about a positive nitrogen balance.

Pfiffner, Vars & Taylor, treating the whole gland and subsequently recovering epinephrine, succeeded in obtaining 75 per cent of the total existing cortical hormone. They also determined the hormone content in several species. By purification, Pfiffner & Vars obtained an amorphous product with a potency equivalent to 200 to 400 dog units per milligram. Wintersteiner, Vars & Pfiffner, by vacuum distillation, obtained inactive crystals.

The primary cause of death following total ablation of the adrenal cortex remains unsettled. According to Swingle *et al.*, a decreased blood volume is the main disturbance produced; the animals show low blood pressure and cannot withstand hemorrhage. The cortical hormone effects a recovery of the blood volume and maintains a well-balanced water equilibrium between blood and tissues. According to Zwemer & Sullivan, water and mineral metabolism are deranged: whereas chlorine and sodium decrease, potassium increases. Zwemer finds that administration of sodium chloride prolongs survival, but Swingle *et al.* deny it. Britton & Silvette, on the other hand, think of a profound and progressive disturbance in carbohydrate metabolism, as shown by hypoglycemia and disappearance of liver glycogen, as being the cause of death in animals deprived of the adrenal cortex. Leloir, Fernández *et al.* and Dambrosi & Leloir, have found after total adrenalectomy: (a) decrease of liver glycogen; (b) decreased glycogen-forming capacity of muscle after glucose injection; (c) delayed and decreased glycogen recovery after fatigue; (d) decreased muscle glycogen. Injection of cortical hormone not only compensates for, but even overcompensates for all these disturbances. Lactic acid formation decreases in the muscles of adrenalectomized guinea pigs (Nachmansohn). Muscle phosphagen is also decreased in adrenalectomized frogs (Moschini). Lundsgaard & Wilson, on the other hand, have found only minor variations of organic phosphorus, phosphagen, hexosephosphate, and adenylypyrophosphate.

Differences continue to be found, depending on the laboratory, as to whether death is a fatal consequence of total adrenalectomy in rats. Histological examination reveals the presence of tiny capsulated islets of cortical tissue scattered in the perirenal fat (Lascano-González). Under certain conditions they may proliferate after adrenalectomy and the animals may thus survive. Cortical extracts permit the survival of rats that would otherwise die after adrenalectomy (Howard & Grollman). Investigators whose adrenalectomized rats show a high mortality find severe disturbances of sexual cycles and the gonads; those, on the other hand, whose rats survive find oestral cycles practically unchanged (del Castillo); neither can they detect variations in the hypophysis concerning its sexual or thyreotropic action (del Castillo). Prolonged administration of cortical extract does not bring about changes of the other endocrine glands (del Castillo, Leloir & Novelli).

If the adrenals are removed in pancreatectomized dogs maintained

under normal conditions through adequate administration of insulin, the blood sugar rises to 0.27 to 0.7 gm. per 100 cc. when insulin is suspended. The glycemia generally remains high, at diabetic levels, falling slowly in some animals in the last days of life (Lewis & Turcati; Leloir). Removal of the pancreas, when an entire adrenal and the medulla of the other have been ablated, produces severe diabetes, the blood sugar rising to 0.36 or 0.38 gm. per 100 cc. (Leloir). Some investigators, however (Barnes *et al.*; Ferrill; Long & Lukens), observed a less severe diabetes when, in addition to the pancreas, one or one and a half adrenals were ablated. Also Hartman & Brownell consider cortin as essential to the maintenance of a high blood-sugar level in diabetes.

*Adrenaline.*—During autolysis of the adrenals, a pressor substance is formed similar to adrenaline, although it is not precipitated by ammonia (Larrain, Roberts & Kunde). Feldberg, Minz & Tsudzimura have found that adrenal secretion, produced by stimulation of the splanchnic nerves, is due to a liberation of acetylcholine<sup>4</sup> in the adrenal medulla. Physostigmine exerts an augmentor effect and high doses of nicotine may abolish it. Acetylcholine would thus be a chemical transmitter of the nervous impulse to chromaffin cells, the splanchnic nerve fibres acting as preganglionic cholinergic fibres (Dale). High doses of atropin decrease or abolish the adrenaline discharge produced by stimulation of the splanchnic nerves (Lewis & Ludueña, confirmed by Houssay & Orías) but do not interfere with the influence of nicotine and quaternary ammonium bases (Lewis & Ludueña). Bronchial dilatation produced by acetylcholine after atropinization is almost entirely due to a discharge of adrenaline (Houssay & Orías).

The work of several investigators has confirmed the increased adrenaline secretion produced by insulin (Yen *et al.*), morphine (Sato & Ohmi), chemical or reflex excitation of the capillaries (Tournade & Rocchisani), asphyxia in the spinal animal (Tournade & Rocchisani), and during bulbo-encephalic anemia (Tournade, Rocchisani & Curtillet). Spinal anesthesia abolishes adrenaline secretion (Tournade & Schotté). Leloir confirmed the observation that nicotine hyperglycemia is due to adrenaline discharge.

The adrenals and the blood of the hedgehog contain more adrenaline when the animal is awake than when it is sleeping; in the rabbit there are diurnal variations (Euler & Holmquist).

<sup>4</sup> Cf. also this volume, pp. 311 *et seq.* (EDITOR.)

Adrenaline, on injection, produces a definite but brief increase of the plasma potassium [D'Silva, confirmed by Marenzi & Gerschman (unpublished data)]. The storage of glycogen produced by insulin during fasting, observed in young rabbits, is due to the adrenaline discharge caused by hypoglycemia (Cope & Corkill). The phosphorus decrease and the lactic acid increase produced by insulin are due to a similar mechanism (Cori & Cori). According to Soskin *et al.*, adrenaline does not interfere with glucose utilization by the muscle.

*Thymus.*—Rowntree, Clark & Hanson have obtained striking results with an acid extract of calf thymus. Its administration through generations augments the weight and hastens the growth of either mature or immature rats, and increases the number and size of the litters. In the fifth generation the growth and development are greatly accelerated: the teeth have erupted and the ears are open on the first day; the eyes are open by the third day; the young mature and breed earlier.

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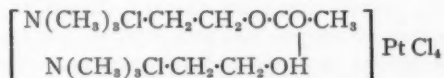
## CHOLINE AND ALLIED SUBSTANCES\*

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### THE CHEMICAL PROPERTIES OF SALTS OF CHOLINE AND ACETYLCHOLINE

Dudley (1, 2) found that when mixtures of the chloroplatinates of choline and acetylcholine were crystallised from water a mixed salt containing one molecule of each base separated from the solution. In comparison with its component chloroplatinates, not only is the mixed salt less soluble in water, but its melting-point is higher, and its crystalline form is strikingly different. The following formula was ascribed to the mixed salt.



The relative insolubility of this mixed salt in water provides a convenient method of separating a small quantity of acetylcholine from a large excess of choline. This method is particularly effective because the excess of choline increases the precipitation of the mixed salt.

Dudley also described a simple method of obtaining a solution of acetylcholine chloride from the chloroplatinate or the chloroaurate. It consists in shaking an aqueous solution of the salt with excess of metallic silver until the solution is colourless. Metallic gold, or platinum, and silver chloride are precipitated and removed, together with the excess of silver, by filtration. The chlorine associated with the basic nitrogen is not removed by the reaction. This method makes acetylcholine chloroaurate available as a convenient source of pure acetylcholine chloride.

A reference to the insoluble "reineckates" of choline and acetylcholine will be found in the section dealing with tissue extracts.

#### CHEMICAL CONSTITUTION AND PHARMACOLOGICAL EFFECTS

Most recent work on the pharmacological actions of quaternary ammonium bases has resulted from the classification of these actions into muscarine-actions and nicotine-actions [Dale (1)].

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*Muscarine-actions.*—These are similar in distribution to the actions of true muscarine (see below). They may be summarised, with some qualification, as a reproduction of the effects of stimulating cranial and sacral autonomic nerves. They are all abolished by small doses of atropine and are unaffected by nicotine. Other drugs having a similar action are pilocarpine, arecoline, etc. Some of the best known examples of this type of action are the fall of mammalian blood pressure due to dilatation of the arterioles (depressor effect), the slowing of the heart, and the contraction of an isolated strip of intestine.

*Nicotine-actions.*—These may be summarized as a stimulation followed by paralysis of autonomic ganglia throughout the body. They are abolished by large doses of nicotine, and are unaffected by small doses of atropine. The liberation of adrenaline and the contracture of voluntary muscles or the muscle of leeches, which these substances cause under certain conditions, are also classed as nicotine-actions. If a large dose of atropine has been injected into a spinal cat the injection of choline or acetylcholine causes a marked rise of blood pressure (pressor effect) which is partly due to the liberation of adrenaline and partly due to stimulation of sympathetic ganglia [see Feldberg & Minz (1), who give references]. This is a typical nicotine-action.

Many of these substances have an action, like that of curare, in blocking the conduction from motor nerves to voluntary muscles. Curare-actions were not specially mentioned by Dale and have been less widely studied than actions of the other two types.

The pharmacological actions of most of the substances discussed below were investigated by Reid Hunt many years ago. In the last few years these substances have been prepared again, and tested for the different kinds of activity indicated above. This classification has been found convenient, since some substances have little or no nicotine-action of any kind, and powerful muscarine-actions of all kinds. It is not, however, safe to assume that because a drug has a marked nicotine-action of one particular kind, all its nicotine-actions will be equally powerful, since examples can be quoted of cases in which this form of argument would lead to false conclusions.

The only accurate way of comparing the action of two of these drugs in any particular test is to give them alternately and adjust the doses until they produce the same effect. The ratio of the doses which have equal effects is taken as a measure of the ratio of the potencies of

the drugs. The chemical formulae of some of the substances which have been studied are given below.

Choline  $(\text{CH}_3)_3\text{N}^+\cdot\text{CH}_2\cdot\text{CH}_2\text{OH}$   
 $\alpha$ -methyl choline  $(\text{CH}_3)_3\text{N}^+\cdot\text{CH}(\text{CH}_3)\cdot\text{CH}_2\text{OH}$   
 $\beta$ -methyl choline  $(\text{CH}_3)_3\text{N}^+\cdot\text{CH}_2\cdot\text{CH}(\text{CH}_3)\text{OH}$   
Muscarine  $(\text{CH}_3)_3\text{N}^+\cdot\text{CH}(\text{CHO})\cdot\text{CH}(\text{C}_2\text{H}_5)\text{OH}$   
Acetylcholine  $(\text{CH}_3)_3\text{N}^+\cdot\text{CH}_2\cdot\text{CH}_2\text{O}\cdot\text{CO}\cdot\text{CH}_3$   
Carbaminoylcholine  $(\text{CH}_3)_3\text{N}^+\cdot\text{CH}_2\cdot\text{CH}_2\text{O}\cdot\text{CO}\cdot\text{NH}_2$

*Choline esters.*—Acetylcholine is better known and has been more widely studied than any other choline ester. Its actions are qualitatively similar to those of choline, but, in almost all types of test, the presence of the acetyl group increases the activity of choline very considerably (1,000 times or more). Muscarine-actions and nicotine-actions are both increased.

Compared with acetylcholine, propionylcholine has much weaker muscarine-actions (the fall of blood pressure in cats and rabbits, and the contraction of rabbit intestine). On the other hand its nicotine-actions on the blood pressure after atropine, and on frog's voluntary muscle, are greater than those of acetylcholine.

In the same tests the muscarine-actions of butyrylcholine were found to be still feeble than those of propionylcholine, and less than 1 per cent of those of acetylcholine, while its nicotine-actions were about the same as, or greater than, those of acetylcholine. In experiments on the blood pressure of rabbits its nicotine-action was so great that it caused a rise of blood pressure even before atropine had been given. Valerylcholine is similar in its action to butyrylcholine, though rather less active. Its nicotine-action on a leech has been found to be remarkably feeble compared with its nicotine-action on frog muscle (*rectus abdominis*) (Simonart, and Chang & Gaddum).

The actions of carbaminoylcholine ("lentin") were first studied by Kreitmair and later by Nöll and Velten. It is at least as active as acetylcholine in various kinds of tests. Its action on blood pressure was stated to be much greater than that of acetylcholine, but this was not confirmed by Chang & Gaddum, who found, in several experiments, that the effect of carbaminoylcholine on the blood pressure was always slightly less than that of acetylcholine. In any case carbaminoylcholine is much more stable than acetylcholine, and is effective when given by mouth. The tests applied by Kreitmair, Nöll, and



Velten were mostly tests for muscarine-activity. Carbaminoylcholine also has nicotine-activity [Feldberg (1)] similar to that of acetylcholine.

*Choline ethers.*—The methyl-, ethyl-, vinyl-, and butyl ethers of choline have been studied by Simonart, who found that their actions were all less than those of acetylcholine. Their nicotine-actions were feeble compared with their muscarine-actions.

*Methylcholines and their esters.*—The introduction of the methyl group in the  $\alpha$ -position in choline or acetylcholine diminishes the depressor muscarine-action on a cat, without affecting the pressor nicotine-action. The introduction of a methyl group in the  $\beta$ -position, on the other hand, diminishes the nicotine-action considerably (Simonart). The substance, acetyl- $\beta$ -methylcholine, was prepared and its properties studied by Hunt (1). The acetyl- $\alpha$ -methylcholine referred to by Hunt in this paper was really acetyl- $\beta$ -choline [Hunt (2)]. Its vasodilator activity was found to be very great, but "could be overcome to a considerable extent by atropine." It was active on either subcutaneous or oral administration. There was some evidence suggesting that its action was potentiated by eserine.

The great activity of acetyl- $\beta$ -choline was rediscovered by Simonart and its actions studied in fuller detail. Acetylcholine has been much used clinically for its vasodilator action, and Simonart was searching for a derivative which would be more suitable for this purpose. He found that acetyl- $\beta$ -choline was not readily hydrolysed by tissues and had a powerful muscarine-action, but that its nicotine-action was very feeble or absent. It was thus very well suited for clinical use, and has since been tested by various investigators and found to be more satisfactory for this purpose than acetylcholine. According to Simonart it produces a fall of blood pressure in cats similar to that due to an equal dose of acetylcholine. Even after the administration of large doses of atropine this depressor effect was never completely abolished and no pressor action was seen. When 1 mg. was placed in the duodenum it caused a secretion of tears and saliva, vomiting, and erection.

Simonart studied the propionyl ester and the ethyl ether of the  $\beta$ -methylcholine and found that they also had powerful muscarine-actions and feeble nicotine-actions, but neither was so well suited as acetyl- $\beta$ -methylcholine for clinical use.

*Muscarine.*—This substance has attracted much interest for many years, not only because of its activity, which is somewhat greater than that of acetylcholine, but also because of its great stability and its

activity when given by mouth, and because of the fact that it has no nicotine-action. It was isolated by Schmiedeberg and Koppe in 1868 from extracts of *Amanita muscaria*, and was later wrongly identified with a substance prepared by treating choline with nitric acid. This substance has often been referred to as artificial muscarine, or even, without any qualification, as muscarine, but it was shown by Dale & Ewins [see Dale (1)] to be really nitrosocholine.

Kögl, Duisberg & Erxleben have now brought forward convincing reasons for ascribing to true natural muscarine the formula given on page 313. It will be seen that muscarine can be regarded as choline with an aldehyde group in the  $\alpha$ -position and an ethyl group in the  $\beta$ -position. The presence of the latter group is of interest because of the work of Simonart, discussed above, which showed that the substitution of a methyl group in the  $\beta$ -position had the effect of depriving acetylcholine of its nicotine-action. It is possible that this ethyl group in muscarine is responsible for the absence of nicotine-action.

#### CHOLINE AND FAT METABOLISM

An interesting action of choline on fat metabolism has been discovered and studied in recent years by Best working with various collaborators. It had been found that large quantities of fat accumulated in the livers of pancreatectomized dogs which were kept alive by regular doses of insulin (Allan, Bowie, Macleod & Robinson). This accumulation of fat could be prevented by giving pancreas by mouth. Later work showed that this effect of pancreas was due to the phospholipids which it contained. Eventually the active agent was identified as choline (Best & Huntsman and Best & Ridout).

Choline also cures fatty livers due to other causes. If rats are fed on a diet containing 40 per cent of beef fat, the fat content of the liver increases (Best, Hershey & Huntsman). This is due to an increase in the neutral fat fraction and can be prevented by adding 0.1 gm. of choline to the daily diet. A diet containing 1 per cent of cholesterol produces an increase of both the cholesterol esters and neutral fat in the livers of rats. Choline prevents both changes. The percentage of phosphatides in the liver is inversely related to the total lipid content, and choline produces an increase in the phosphatide content which is probably secondary to the decrease in total lipoids (Best, Channon & Ridout). Choline also prevents the accumulation of fat in the liver during starvation. This inhibition of the accumu-

lation of fat in the liver is the only effect which choline has been shown to have on the fat metabolism. Fat depôts in other parts of the body appear to be unaffected. The effect apparently is not due to an action on the absorption or excretion of fat, and the most probable explanation is that choline stimulates the liver to oxidise both neutral fats and cholesterol esters.

The effect is produced when choline is injected subcutaneously, but does not appear to be related to the purely pharmacological effects of choline. Acetylcholine is no more active than choline itself. Betaine produces the same effect. These results were reviewed by Best in June, 1934.

#### THE METABOLISM OF CHOLINE ESTERS

There are two good reasons for knowing that the acetylcholine which is sometimes found in tissue extracts is not derived from acetylcholine freely diffusible in the tissue. In the first place free acetylcholine would rapidly be destroyed by the cholinesterase which is present both in the blood and in the other tissues. In the second place the amount of activity present in the extract is often much more than sufficient to stimulate the tissue to maximal activity. Two theories have been suggested as to the way in which acetylcholine is stored in tissues. It may either be present as an active and comparatively stable precursor, or else it may be stored in some special organ which prevents it from being freely diffusible.

It has been suggested that the acetylcholine is formed in tissues by the acetylation of choline, and evidence has been obtained that in the presence of tissue extracts and a large excess of choline and acetate, a certain amount of synthesis of acetylcholine may take place (Abderhalden & Paffrath, and Ammon & Kwiatkowski). According to Beznak, acetylcholine is formed by the minced tissue of a frog's heart in the presence of sufficient eserine to inhibit its destruction.

Our knowledge concerning the destruction of acetylcholine is more complete. Acetylcholine is rapidly hydrolysed in the presence of alkali to choline and acetate. The maximum stability is at pH 3.9. Hoffmann boiled a solution buffered at pH 3.9 for twelve hours and found by titration that only 3.8 per cent of the original acetylcholine was hydrolysed. In the presence of blood or extracts of other tissues hydrolysis occurs very rapidly under the action of a specific cholinesterase [Dale (1), Loewi & Navratil, Engelhart & Loewi, Stedman, Stedman & Easson, etc.].

The amount of hydrolysis can be measured either by pharmacological tests (Matthes), or by chemical methods. Stedman, Stedman & Easson measured the activity of the enzyme by adding a large excess of the substrate and keeping the activity constant by continuous titration. In Ammon's experiments the acetate liberated by hydrolysis combined with base and liberated carbon dioxide from carbonates; the amount of carbon dioxide was measured in a Warburg apparatus.

Purified preparations of cholinesterase were made by Stedman, Stedman & Easson from horse serum. Although these purified preparations had a marked effect on acetylcholine and butyrylcholine, their action on methylbutyrate was comparatively feeble compared with that of the original serum. These results show that the action of the original serum on methylbutyrate is due to a different enzyme, and strengthen the view that cholinesterase is specific for choline esters.

The amount of cholinesterase in different organs (Plattner & Hintner) or in the blood serum of different species varies widely. Stedman, Stedman & White were unable to detect any activity at all in serum obtained from many animals (such as the frog, rabbit, rat, or ox). On the other hand Galehr & Plattner, and Plattner & Bauer, using pharmacological tests, found that whole blood from some of these species did have cholinesterase activity. The activity appears to be more concentrated in the cells than in the serum or plasma (Galehr & Plattner). Observers are agreed that the activity of human blood or serum is very great. At 40° acetylcholine is almost completely destroyed by human blood in fifteen seconds.

Eserine (physostigmine) has a powerful inhibitory action on cholinesterase and some inhibitory action on other esterases. A preliminary injection of eserine increases the pharmacological effect of the active choline esters in every case in which the experiment has been tried. The inactivation of cholinesterase is a slow process, and may take more than fifteen minutes when the concentration of eserine is low. The action is reversible, since the enzyme regains its activity when the eserine is removed by dialysis (Matthes). A probable theory of this inhibition has been put forward by Stedman & Stedman, who suggest that eserine, which is itself a stable ester, acts by combining with the enzyme in the same way, and by the same mechanism, as the choline esters, thus blocking the enzyme.

Cholinesterase is also inhibited by miotine, and other urethanes chemically allied to eserine and having similar pharmacological actions (Matthes; White & Stedman; and Aeschlimann & Reinert).

THE PHARMACOLOGICAL ESTIMATION OF ACETYLCHOLINE  
AND CHOLINE

The best pharmacological methods for estimating the amount of acetylcholine in a solution have an error of 5 or 10 per cent, but they are simple to carry out and are much more sensitive than any chemical method. A millionth part of a milligram of acetylcholine can be detected under suitable conditions; if 1 $\gamma$  (0.001 mg.) of this substance is available it is possible to carry out a series of quantitative tests and to discriminate between effects due to acetylcholine and those due to other closely related choline esters. Similar quantities of choline can be estimated by acetylating the solution and then applying a pharmacological test for acetylcholine.

The most sensitive and specific tissue which can be used in a test as a pharmacological reagent for acetylcholine is the longitudinal muscle from the anterior end of the dorsum of a leech (*Hirudo medicinalis*) suspended in a salt solution containing eserine (Minz). Acetylcholine can also be estimated by its effect on isolated rabbit's auricle, frog's heart, the small intestine of a mouse or rabbit, frog's voluntary muscle (*rectus abdominis*), cat's blood-pressure, or cat's voluntary muscle sensitized by preliminary section of its motor nerve. In this list the tests are given in descending order of sensitivity. The relative values of these different tests have been discussed in papers by Chang & Gaddum, Kahlson, Beznak, etc.

None of these tests is completely specific for acetylcholine, and before concluding that any particular effect is due to this substance it is necessary to carry out special confirmatory tests of some kind.

Muscarine-actions are abolished by atropine, and nicotine-actions by nicotine. The effects of the solution under test should be compared with those of acetylcholine before and after the administration of these drugs.

Acetylcholine, like other active unstable choline esters, has the following properties, and tests should be carried out to see whether the unknown substance resembles acetylcholine in these respects.

- (a) Many of its effects are increased by eserine.
- (b) It is rapidly hydrolysed by blood, or purified cholinesterase, and this destruction is inhibited by eserine.
- (c) It is unstable in alkaline solutions and very stable in weakly acid solutions. If a portion of a solution is mixed with an equal volume of 2N NaOH and left for ten minutes at room temperature any acetylcholine is destroyed and choline is unaffected.

Such special tests distinguish active choline esters from choline, but they do not distinguish acetylcholine from other choline esters. The only way in which this can be done is to carry out quantitative comparisons, by a series of different methods, of the unknown solution and a known solution of acetylcholine (Chang & Gaddum). The evidence discussed, dealing with the activity of choline esters in different tests, shows that if the activity is due, for example, to propionylcholine the quantitative results of such comparisons will differ widely among themselves. If the activity is entirely due to acetylcholine in every case, then the results of the tests must agree within the error of the tests themselves.

#### TISSUE EXTRACTS

*Choline.*—Since choline was first obtained from pig's bile by Strecker in 1848 it has been isolated from a great variety of fluids obtained from tissues. Much of this choline is derived from lecithin by autolysis. A litre of blood plasma may normally contain 160 to 300 mg. of choline combined as lecithin (Lintzel & Monasterio). A litre of serum is said to contain 5 to 10 mg. of free choline, but it is difficult to be certain that this has not been liberated from lecithin *post mortem*. The quantity of choline in tissue extracts derived from the hydrolysis of choline esters must be comparatively small.

Strack, Neubauer & Geissendörfer have obtained results which suggest that much of the evidence for the presence of free choline in tissues is unreliable owing to delay in preparing the extracts. They found that when dogs' livers were extracted with alcohol as rapidly as possible after death, the extracts contained 0 to 43 mg. of choline per kg. of liver. Similar extracts prepared five hours after death contained 136 to 164 mg. of choline per kg. of liver. A similar gradual formation of free choline occurred in experiments in which the tissue was cut in slices and soaked in alcohol. These experiments show that alcohol is not a suitable extractive for use in experiments designed to measure the free choline in tissues.

Heesch believed that an extract prepared with trichloroacetic acid contained only the choline which had originally been free and none of that derived from lecithin. He found that such an extract of blood serum had 2.5 to 10 mg. of choline per litre of blood, and that the figure was unaffected by preliminary shaking of the blood serum with ether in order to remove the lecithin.

In the experiments of Wrede & Bruch the original extract was prepared with hot acidulated water and the choline in this extract was isolated and weighed as the chloroaurate. This was taken to represent the free choline in the tissue.

Lintzel & Monasterio estimated the lecithin-choline by treating the ether-soluble fraction with permanganate in the presence of formaldehyde. The choline was oxidised to trimethylamine, which was then distilled into acid and estimated by titration.

*Acetylcholine.*—Pharmacological evidence for the presence of a choline ester in tissues had been obtained by various workers, when Dale & Dudley (1) succeeded in 1929 in isolating acetylcholine from an extract of horse spleen in sufficient quantities for chemical identification. Since that time the choline esters in tissues have been widely studied, but all the evidence available is compatible with the view that acetylcholine is the only ester which occurs in the body.

Dale & Dudley found that the spleen could be removed from the horse and kept for hours without appreciable loss of acetylcholine, but as soon as the tissue was cut up for extraction, acetylcholine rapidly disappeared. In order to obtain maximal yields the tissue must be disintegrated beneath the surface of some liquid which will inhibit the disappearance of the acetylcholine. Dale & Dudley used alcohol as an extractive, but Chang & Gaddum found that extracts prepared with trichloroacetic acid contained 10 to 30 per cent more activity than alcoholic extracts. Recent evidence (Barsoum) has shown that if, after the removal of the alcohol, alcoholic extracts are treated with the trichloroacetic acid, their pharmacological action on the leech is increased, so as to be equivalent to that of an extract prepared directly from the same tissue with trichloroacetic acid alone. Some of the acetylcholine in the original alcoholic extract thus appears to be present in a form which prevents it from producing its characteristic effects.

Bischoff, Grab & Kapfhammer (1), and Kahlson have used metaphosphoric acid in the preparation of extracts which were subsequently tested for acetylcholine. This method has the advantage that the pH of the extract is approximately that at which acetylcholine is most stable.

Kapfhammer & Bischoff [see also Bischoff, Grab & Kapfhammer, and Dudley (3)] have described a very effective method of isolating choline, or acetylcholine, from tissues. The tissue is extracted with alcohol, and purified with trichloroacetic acid. The important part of



the purification depends on the fact that choline and acetylcholine form very insoluble "reineckates" in the presence of Reinecke's salt— $[(\text{NH}_3)_2\text{Cr} \cdot (\text{CNS})_4]\text{NH}_4$ . The precipitate of reineckate may be thoroughly washed with ice-cold water and alcohol, which dissolves the reineckates of other substances, but not those of choline and acetylcholine. The precipitate which has been purified in this way can be dissolved in acetone and treated with aqueous silver sulphate solution, which precipitates silver reineckate, and liberates choline and acetylcholine. Barium chloride is then added in a quantity equivalent to the quantity of silver sulphate used, and the silver chloride and barium sulphate are removed in a centrifuge. The chloroaurates of choline and acetylcholine are precipitated from the supernatant fluid by adding gold chloride. The method of separating these chloroaurates from one another is discussed above (page 311).

Bischoff, Grab & Kapfhammer applied this method of extraction, and announced the isolation of unexpectedly large quantities of acetylcholine from blood and various other tissues. Their results have not been universally accepted. Their first paper was concerned with ox blood and numerous attempts have been made to confirm their results with this tissue. Vogelfanger obtained results supporting their conclusions, but all the other evidence is in conflict with them [Wrede & Keil, Dale & Dudley (2), Dudley (3), Chang & Gaddum, Gollwitzer-Meier, Kahlson, Kahlson & Römer, Ettinger & Hall, and Loach]. This work was undertaken for the specific purpose of confirming the conclusions of Kapfhammer & Bischoff with regard to blood, and in several cases the technique used by these workers was followed in all its details.

Bischoff, Grab & Kapfhammer had found up to 40 mg. of acetylcholine per litre of blood. The largest amount detected by the above workers was less than 0.1 mg. per litre. The weight of evidence is thus against Bischoff, Grab & Kapfhammer, but no adequate explanation of the discrepancy has been suggested.

A number of papers have been published in recent years dealing with the pharmacological evidence for the presence of acetylcholine in different tissues [Chang & Gaddum (who give references to most of the earlier work), Chang & Wong, Kahlson, Dikshit, Plattner, Beznak, Barsoum].

Surprisingly large quantities of acetylcholine are present in extracts of the spleens of horses and oxen (4 to 30 mg. per kg.). The spleens of most laboratory animals contain little or none ( $< 0.1$  mg.

per kg.). Human placenta also contains very large quantities (15 to 133 mg. per kg.). Apart from these two exceptional tissues the highest concentrations have been found in the trunks of the cholinergic [Dale (3)] nerves (Barsoum), and in the tissues in which cholinergic nerves run. The concentration in the rabbit's auricle is high (4 mg. per kg.) compared with that in the ventricle [Engelhart (1)], and that in the horse's suprarenal medulla (0.45 mg. per kg.) is about three times that in the suprarenal cortex (Feldberg & Schild). The concentration of choline, on the other hand, is higher in the cortex than the medulla. In these and other ways the distribution of acetylcholine conforms roughly with expectation, but there are several minor anomalies, and it cannot be said that the correlation between the distribution of acetylcholine and that of cholinergic nerves is very close.

#### THE RELEASE OF CHOLINE ESTERS BY NERVES

Evidence that various nerves produce their effects by liberating chemical substances has been accumulating rapidly in recent years. A number of reviews on this subject have been published [Dale (2, 4, 5), Cannon (1, 2), Frédéricq, Parker].

These nerves can be described in two main classes according to the nature of the substance which they liberate. A convenient nomenclature has been suggested by Dale (3). *Cholinergic* nerves are nerves, such as the vagus, the effects of which are transmitted by acetylcholine, or some similar substance. *Adrenergic* nerves are nerves the effects of which are transmitted by the liberation of adrenaline, or some similar substance. The present review is concerned only with cholinergic nerves.

Chang & Gaddum proposed the term "A.C.-substance" to mean a substance pharmacologically identified as acetylcholine. They also proposed "A.C.-equivalent" to mean "the apparent concentration of acetylcholine in terms of the chloride." In some of the experiments discussed below the pharmacological evidence was such as to leave little doubt that the A.C.-substance was actually acetylcholine. In other cases the evidence was less complete. The terms proposed by Chang & Gaddum are applied to both classes of result. There is at present no purely chemical evidence identifying the A.C.-substance liberated by nerves, and it is doubtful whether sufficient material will ever be available for such evidence to be obtained.

The most convincing evidence of a cholinergic mechanism is the

observation that stimulation of the nerve causes the appearance of A.C.-substance in the fluid bathing the tissue. The discovery that eserine protects choline esters from destruction in tissues has greatly facilitated such experiments. The experiment is often quite simple when eserine is present to preserve the A.C.-substance, but gives negative results in the absence of this drug.

In many cases it has been found that the injection of eserine greatly increases the normal physiological effects of nervous stimulation. Even in the absence of the other evidence, such an observation strongly suggests a cholinergic mechanism (Dale & Gaddum). When it has been proved that A.C.-substance is actually liberated, the observation that the physiological response is increased by eserine supports the view that the A.C.-substance plays an important part in the transmission of the nervous impulses and is not merely an incidental by-product.

*Parasympathetic nerves.—*

*Heart:* The advances which have been made recently in our knowledge of the chemical transmission of nervous impulses began with the work of Loewi *et al.*, who showed that stimulation of the vagus nerve to the isolated frog heart caused the liberation of an A.C.-substance (*Vagusstoff*) into the salt solution bathing the heart. A review of criticism of this observation has been published by Hirschberg. It is now generally agreed that the observation was correct. Various improvements in the original technique have been suggested [see, for example, Bain (1)] and the original observation has been confirmed in many ways.

Attempts to demonstrate the liberation of *Vagusstoff* when blood is flowing through the heart in the normal way have often been unsuccessful, probably because blood very rapidly destroys *Vagusstoff* (Galehr & Plattner). On the other hand Hansen & Rech obtained convincing results in experiments with pregnant guinea pigs. They recorded the electrocardiograms of both mother and foetus, and stimulated the maternal vagus. This produced a slowing of the foetal heart which Hansen & Rech attributed to the passage of *Vagusstoff* through the placenta to the foetal circulation. Feldberg & Kraye obtained very convincing evidence of the liberation of A.C.-substance by using eserine to inhibit its destruction by blood. Various workers have obtained evidence that the cardiac *Vagusstoff* also resembles acetylcholine in causing a contraction of isolated intestine [Brinkman & Van Dam, Jendrassik, and Bain (1)].

These results confirm previous observations (Witanowski, Loewi & Navratil, etc.) which suggested that *Vagusstoff* was an active choline ester. Since acetylcholine is the only ester that has been isolated from animal tissues it is highly probable that *Vagusstoff* is acetylcholine. Pharmacological comparisons which would differentiate choline esters from one another have been applied to the substances liberated by various other nerves discussed below, and in every case the result has been consistent with the view that the substance was acetylcholine. There is no reason to doubt that similar results would be obtained in experiments with cardiac *Vagusstoff*.

*Stomach and intestine:* Early work on the importance of choline and choline esters for the activity of the intestine was reviewed by Magnus. Since then evidence has been obtained that extracts of intestine contain A.C.-substance (Chang & Gaddum). It has also been shown that if sufficient eserine has been injected, the portal blood coming from the stomach or intestine contains detectable quantities of A.C.-substance. The A.C.-equivalent of the portal blood is increased by stimulation of the vagus. The A.C.-substance in the fluid collected from a perfused stomach during stimulation of the vagus is almost certainly acetylcholine itself [Feldberg & Rosenfeld, and Dale & Feldberg (1)].

*Lungs:* The pulmonary fibres of the vagus nerve would be expected from previous work to be cholinergic. Direct evidence in support of this view has been obtained by Thornton (see also Saalfeld).

*Eye:* Engelhart (2) treated a rabbit's eye with eserine and then exposed it to light. This caused the appearance of A.C.-substance in the aqueous humour. The substance was presumably liberated by the oculomotor nerve.

*Salivary glands:* In 1932 evidence was obtained almost simultaneously by several groups of workers that the parasympathetic nerves running in the chorda to the submaxillary glands are cholinergic [Babkin, Stavratsky & Alley, Henderson & Roepke (1, 3), and Gibbs & Szelöczy]. The liberation of A.C.-substance was demonstrated both in perfusion experiments and in experiments with intact circulation. The tests applied would not differentiate between different choline esters, but there is no reason to doubt that in these experiments the glands were liberating acetylcholine at the rate of about 0.05 to 0.2  $\gamma$  per minute. On reinjection the fluid caused the secretion of about half the quantity of saliva that had been secreted as the result of the original stimulus.

*Urinary bladder:* Henderson and Roepke (4) perfused the bladder and found that stimulation of the pelvic nerve caused the liberation of A.C.-substance.

*Nervi erigentes:* The powerful vasodilatation which causes erection of the penis is probably controlled by cholinergic fibres running in the *nervi erigentes* [Henderson & Roepke (2)].

*Sympathetic postganglionic nerves.*—In all the cases that have been tested, sympathetic postganglionic nerves contain adrenergic fibres, but there is evidence that many of them also contain cholinergic fibres. The first clear evidence on this point came from a study of the nerves which cause vasodilatation and pseudomotor contractures in voluntary muscle. This work is discussed in a separate section below.

There is evidence that the sweat glands in a cat's foot are controlled by cholinergic fibres running in the sympathetic nerves to the leg [Dale & Feldberg (2)]. The sweat glands in man are presumably controlled in the same way, but those of certain other animals, such as the horse, are probably not. The pharmacological reactions of these glands in the horse suggest that they are controlled by adrenergic fibres.

The uterus contracts in the presence of drugs having a muscarine-action, but its nerve supply comes from the sympathetic through the hypogastric nerves. Evidence has recently been obtained that the hypogastric nerve of a bitch contains cholinergic fibres running to the uterus (Sherif).

*Nerves causing vasodilatation and pseudomotor contractures in voluntary muscles.*—About a week after section of the motor nerves, mammalian voluntary muscle becomes abnormally sensitive to acetylcholine. The injection of this substance, or of other similar substances, then causes a slow pseudomotor contracture of the muscle. Stimulation of the vasodilator nerves to such sensitized muscles causes a similar contracture, and it has been suggested that this is due to the fact that such vasodilator nerves normally act by liberating acetylcholine, or some similar substance, and that a certain amount of this substance reaches the muscle fibres, so that, if they have been sensitized, they contract. The evidence for this hypothesis was discussed in detail by Dale & Gaddum. More recently evidence has been obtained that stimulation of the vasodilator nerve to the tongue causes the appearance of A.C.-substance in the venous effluent [Bain (2, 3) and Feldberg (2, 3)]. This substance was probably liberated by vaso-

dilator nerves, but it might possibly have come from the secretomotor fibres to the mucous glands on the surface of the tongue.

The anatomical nature of the nerves producing these effects varies in different parts of the body. The vasodilator nerves to the tongue run in the chordalingual and are parasympathetic. On the other hand typical sympathetic nerves supply vasodilator fibres to the buccal mucous membrane of a dog, and pseudomotor fibres to the canine muscle which runs along a dog's upper lip. These fibres are probably cholinergic (Euler & Gaddum).

The nerves causing vasodilatation and pseudomotor contractures in the muscles of limbs were at one time thought to be antidromic fibres running in the dorsal roots of spinal nerves. Hinsey & Cutting have, however, come to the conclusion that those causing the pseudomotor contractures, at any rate, are typical sympathetic nerves. Independent evidence of the presence of cholinergic vasodilator fibres in the sympathetic nerves to a limb has been obtained by Bülbring & Burn.

*Preganglionic sympathetic nerves.*—There is evidence that the nerve fibres in the splanchnic nerves which produce a secretion of adrenaline from the suprarenal medulla are cholinergic, so that the liberation of adrenaline when these nerves are stimulated is a secondary consequence of the liberation of A.C.-substance [Feldberg & Minz (2), and Feldberg, Minz & Tzudzimura].

The nerve fibres running in the cervical sympathetic to the superior cervical ganglion are also cholinergic. Kibjakow introduced a technique for perfusing this ganglion in a cat with Locke's solution. He collected the outflowing fluid and tested it by perfusing it through a second ganglion. He found that fluid collected during stimulation of the cervical sympathetic had a stimulant action on the second ganglion shown by a contraction of the nictitating membrane. Feldberg & Gaddum failed to confirm this observation, but found that fluid collected under similar conditions in the presence of eserine contained an A.C.-substance which was almost certainly acetylcholine (see also Feldberg & Vartiainen). Similar results were obtained by Barsoum, Gaddum & Khayyal, using the inferior mesenteric ganglion of a dog.

*Motor nerves to voluntary muscle.*—Evidence has been obtained by Dale & Feldberg (2) which suggests that the motor nerves to voluntary muscle may be cholinergic.

*Nerves and the acetylcholine in extracts.*—The A.C.-equivalent of the fluids obtained in such experiments during nervous stimulation is

very small compared with the A.C.-equivalent of extracts of tissues. It might therefore be expected that stimulation would produce no change in the A.C.-equivalent of extracts. According to the most recent and convincing evidence, this is indeed the case, but earlier results suggested that stimulation caused a large increase in the A.C.-equivalent of extracts of tissues. Work on this subject has been discussed by Vartiainen, and by Beznak.

Chang & Gaddum found that degenerative section of nerves caused some, but not all, of the A.C.-substance to disappear from tissues. Beznak failed to confirm this observation.

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## VITAMINS\*

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A rapid preliminary survey of some of the principal developments in the year's work<sup>1</sup> will serve the double purpose of introduction and summary.

In the "B" group, interest has centred largely on flavin. Flavin is found not to be the anti-pellagra factor, but is one constituent of a complex "vitamin B<sub>2</sub>." Its chemical structure has been intensively investigated, and the climax has been reached in the synthesis of a flavin-like substance possessing biological activity.

The P-P factor for human beings is confirmed by further direct clinical tests to resemble, in its distribution, the rat (or dog) anti-pellagra factor, present in the B<sub>2</sub> complex. The identity of certain anaemia-preventing factors is also being narrowed down.

The conviction grows that the crystalline preparations of vitamin B<sub>1</sub> represent the vitamin in a virtually pure condition. Its relation to carbohydrate metabolism is being further analysed. A convenient new method of assay, based on electrocardiographic measurements, has been described. Difficulties about "vitamin B<sub>4</sub>" are increased by the abandonment of the method of estimating it.

Vitamin C has been discovered in various unsuspected sites, e.g., in the wall of the gut. It is found to activate certain enzyme systems, but this influence may be unspecific. Its action on tooth structure has been elucidated. In experimental animals, partial deficiency of vitamin C may provoke a "rheumatic tendency"—an observation of possible clinical suggestiveness.

Vitamin A. Skin lesions are now stressed as an important clinical feature of hypovitaminosis A, prevalent in many parts of the world. The incidence of night blindness in some regions has also been shown to be surprisingly high. Details of the chemistry of the "vitamin-A cycle" in the eye have been published. The criterion of

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<sup>1</sup> It should be mentioned that the literature continues to be so vast that we have had space to refer to no more than about one paper out of every five published, and a number of interesting and significant contributions have unfortunately had to be left unmentioned.

vitamin-A activity in a carotenoid pigment or derivative is the presence of a  $\beta$ -ionone ring attached to four conjugated double bonds. In maize the activity is due not to carotene but to a related pigment, cryptoxanthin.

Differences between the behaviour of vitamin D in natural sources and in the form of calciferol remain to be elucidated. The precise position of the double bonds and of the hydroxyl group in ergosterol is in dispute, otherwise general agreement has been reached as to the structural skeleton of the provitamin.

Clinical trials with vitamin E indicate possible practical applications.

Vitamin F (= essential fatty acids) prevents sterility of a peculiar type, marked by impairment of the birth mechanism.

New International Standards have been accepted for vitamins A and C.

### VITAMIN B<sub>1</sub> (B)

*Incidence of beri-beri in humans.*—A number of clinical surveys which have appeared during the year illustrate the continued prevalence of a preventable disease. In the Straits Settlements—by no means one of the worst areas—over 700 deaths from beri-beri have been reported during the past ten years. At Nanking, Young & Huang give an analysis of 109 patients admitted to the Central Hospital in 1930–1932. In the Westman Isles, Iceland, beri-beri is on the increase, as the result of the growing use of white flour and polished rice in place of rye and barley and the decreased use of milk (Kolka). Dumont gives a description of twenty-seven cases seen among Congo natives.

In Palestine, lack of vitamin B appears to be the cause of a condition commonly met with in babies resembling summer diarrhoea. It is said to respond very effectively to vitamin-B therapy (Grünfelder).

*"Secondary beri-beri."*—As met with outside the endemic belts, this disease may be attributed to gastro-intestinal disturbances. Strauss has analysed a series of such cases. Alcoholism is the most usual cause but persistent vomiting, coeliac disease, dysentery, and carcinoma of the stomach are all sometimes responsible (cf. Wechsler). In Strauss's experience the polyneuritic symptoms disappeared readily when vitamin B was injected, or when large doses were fed.

*Clinical hypovitaminosis-B.*—Evidence has been accumulating for some years that children in America or Western Europe may fre-

quently receive sub-optimal allowances of vitamin B. Recently Summerfeldt has shown convincingly, in a well controlled experiment, that a group of children given extra vitamin B, in the form of wheat germ, cereals, and yeast, made three to four times the expected gain in weight. Crimm and his coworkers rather similarly concluded that the addition of extra vitamin B to the diet of a number of tuberculous children was responsible for a marked increase in weight gains. Again Brown and his associates have tested the therapeutic action of a daily dose of 3 gm. of yeast in a group of 351 underfed women and children from economically low classes, and in almost all cases benefit was observed, either in appetite, in weight gains, or in bowel function. Jung calculates the daily need of an adult to be from 200 to 300 daily rat doses.

*The vitamin-B needs of insects and of various species.*—The vitamin-B requirements of insects appear in many ways to resemble those of the higher animals. Hobson has given a detailed description of his experiments on blow flies, proving that their larvae are unable to grow in sterile blood without the addition of vitamin-B complex (given in the form of yeast autolysate). He confirms the suggestion made by Wigglesworth in 1929 that the function of the symbiotic micro-organisms found in the blood stream of blood-sucking insects is the synthesis of vitamin B. Vitamin B<sub>1</sub> has also been shown to be necessary for the development of cockroach larvae (Moskalenko)—which are even recommended for use as test animals in vitamin determinations.

A novel paper by Scheunert tells us that the so-called "star-gazing" disease of lions (a head retraction) is due to vitamin-B deficiency, and responds readily to yeast. He believes that the vitamin-B needs of carnivores in captivity are often not sufficiently recognised.

*Lesions of vitamin-B<sub>1</sub> deficiency.*—A histological study by Prickett has led him to the conclusion that the lesions responsible for the nervous symptoms in polyneuritis lie in the central rather than in the peripheral nervous system. This finding seems to accord well with what is known of the biochemical abnormalities, the diminished oxygen uptake being more readily demonstrated in the brain than in other tissues examined (*vide infra*).

Babkin has made an interesting study of the effect of vitamin deficiency on the nervous control of the gastric secretion. He found that in avitaminosis there was a definite impairment in the secretory response to sham feeding, to the subcutaneous injection of histamine,

or to the introduction of food or alcohol into the small intestine. When vitamin B (yeast) was given the response was restored to normal. Similarly Cowgill & Gilman have found a diminution of gastric secretion in dogs starved of vitamin B. Sure & Thatcher report gastric ulcers in rats arising as a result of specific deficiency in vitamin B<sub>1</sub>.

*Crystalline vitamin B<sub>1</sub>.*—In recent volumes the reviewer has expressed the opinion that the crystalline specimens obtained by Jansen & Donath, by Windaus, by Peters, by Otake, by Van Veen (1) and by others all represent, essentially, the pure vitamin itself with the admixture only of a small and variable amount of inactivated vitamin. In spite of certain dissentient views, the weight of opinion now seems to favour this conclusion (cf. Van Veen; Kinnersley, O'Brien & Peters; Otake).

Otake finds that a crystalline preparation from bakers' yeast is identical in every way with one obtained from rice polishings. The analysis corresponded with C<sub>12</sub>H<sub>16</sub>N<sub>4</sub>SO<sub>2</sub> or C<sub>12</sub>H<sub>18</sub>N<sub>4</sub>SO<sub>2</sub>, and the preventive rat dose was 1.0 γ, or the pigeon dose, 2.5 γ.

*Concentration processes.*—Williams, Waterman & Keresztesy describe a process by which a yield of crystalline vitamin B<sub>1</sub> may be obtained several fold larger than that previously reported. The recovery is about 25 per cent. A novel feature of their process is the extraction of the adsorbate on fuller's earth with quinine sulphate in place of the barium hydroxide usually used. The latter was found to be relatively inefficient.

*Physicochemical properties of vitamin B<sub>1</sub>.*—Heyroth & Loofbourow have examined the light-absorption curves of various crystalline specimens and confirm the fact that the activity is correlated with a maximum at 260 mμ. Their observations lead them to suggest that the active molecule is built around a pyrimidine or cytosine type of nucleus. Discrepancies previously noted with Peters' specimen were attributable to the solvent employed. With Windaus's preparation, however, the absorption curve seems different, and the interesting suggestion is thrown out that more than one pyrimidine base can form the nucleus of the active molecule, uracil serving in Windaus's crystals and cytosine in the others.

An interesting note by Keenan & Kline draws attention to the observation that vitamin B<sub>1</sub>, although readily inactivated at 100° by moist heat, can be heated in the dry condition to temperatures as high as 120° for twenty-four hours or more without change.

It may be recalled that in the past some controversy has centred



around the question whether active fractions of vitamin B<sub>1</sub> gave the Pauly reaction for an iminazole group. In 1931 Peters came to the conclusion that the vitamin gave a positive test, provided a suitable degree of alkalinity were employed. By the use of formaldehyde as a stabilising agent, combined with the presence of alkali, a test of high specificity is now reported (Kinnersley & Peters).

*Determination of vitamin B<sub>1</sub>.*—An account has appeared of the use of Harris's electrocardiographic method for the routine estimation of vitamin B<sub>1</sub> (Birch & Harris). The principle of the method is to feed graded doses of the material under test to a series of depleted rats and then measure the effect on the heart beat during a short period afterwards. The duration of the effect is proportional to the dose given. The authors give a detailed survey of results obtained by this method as compared with other standard procedures. By its use differences of 20 per cent in activity can be readily detected. The method has been proved to be specific since pure crystalline preparations of vitamin B<sub>1</sub> give the response while other vitamin-B fractions do not. This method has a number of advantages over others; e.g., it can be used for foodstuffs containing only moderate amounts of vitamin B, which cannot be readily tested by the curative pigeon method or by the cure of convulsions in rats. Again it could be used for cereal products, which in the authors' experience frequently give rise to refection when tested by the rat-growth method.

*Distribution of vitamin B<sub>1</sub>.*—Some sixty papers have appeared during the year describing either the distribution of vitamin B<sub>1</sub> in various foodstuffs, or the resources of different geographical areas, or the effects of different treatments, etc. The vitamin-B<sub>1</sub> activities of forty common Bengali foodstuffs have been measured by Ghosh & Guha. Harris has found that soil treatment has surprisingly little effect on the percentage of vitamin B<sub>1</sub> in wheat. Vogt-Møller has examined wheat milling from the nutritional point of view. Van Veen (2) advocates the use of unwashed, slightly milled rice in districts of beri-beri endemicity, for he finds that washed rice has a greatly diminished activity, while unmilled rice is generally disliked.

*Mode of action of vitamin B<sub>1</sub>.*—As we have recorded in past years, work from various quarters has indicated that vitamin B<sub>1</sub> is concerned in carbohydrate metabolism; more specifically, that in the absence of the vitamin, lactic acid accumulates in the animal body instead of being disposed of as normally. Peters and his coworkers, who have examined the action of the vitamin upon lactic acid metab-

olism *in vitro*, have published some further interesting results during the past twelve months. It will be recalled that their past work showed that avitaminous brain tissue to which lactic acid was added as substrate failed to take up oxygen normally. When vitamin B<sub>1</sub> was added to this preparation the oxygen uptake was partly restored, even although the excess of lactic acid was not removed thereby. Thompson has now shown that kidney behaves similarly, while other tissue examined did not exhibit this effect. The respiratory quotient of brain tissue from an avitaminous pigeon is also low in presence of lactate, and is raised on the addition of vitamin B<sub>1</sub> (Sinclair).

It has now been found by Peters & Sinclair that pyrophosphate takes some part in the reaction; in other words, the three substances, vitamin B<sub>1</sub>, lactate, and pyrophosphate, appear to form a coupled oxidation system. The details of this mechanism still remain vague, but a new turn has been given to the investigations by the finding of Peters & Thompson that pyruvic acid is formed by avitaminous pigeon brain (but not by normal) during its respiration in lactate solution, and that this pyruvic acid largely disappears upon the addition of vitamin B<sub>1</sub>. A special interest attaches to these observations in view of the theories of Embden and of Meyerhof as to the rôle of pyruvic acid as a normal intermediate in carbohydrate metabolism.

The suggestion was made some years ago by Harris that the low heart rate observed in rats suffering from vitamin-B<sub>1</sub> deficiency was to be correlated with an accumulation of lactic acid, and evidence in confirmation of this suggestion has since been obtained (Birch & Harris).

Birch & Harris speak of vitamin B<sub>1</sub> as being a coenzyme-like substance, intervening in the chain of carbohydrate-oxidation reactions. Following up this side of the problem, Birch & Mann have shown that two separate coenzymes are needed for the dehydrogenation of lactic acid. One of these exhibits some temporary curative action in vitamin-B<sub>1</sub> deficiency. Further analyses of these complex enzyme systems are needed to narrow down the specificity of the vitamin reaction.

The occurrence of methyl glyoxal in the urine of polyneuritic dogs and rats, and in the urine and cerebrospinal fluid of infants suffering from "acute toxic dyspepsia" [apparently a manifestation of vitamin-B deficiency (Geiger & Rosenberg)], gives further evidence of a disordered carbohydrate metabolism in the absence of vitamin B<sub>1</sub>. So also does the increased content of glycogen in the liver of poly-

neuritic pigeons, which has been confirmed by several workers (Schrader; Abderhalden & Wertheimer; Ariyama).

### THE VITAMIN-B<sub>2</sub> COMPLEX

*Composite nature of vitamin B<sub>2</sub>.*—By definition, vitamin B<sub>2</sub> is the more heat-stable, water-soluble dietary factor, recently described and named P-P ("pellagra-preventive") factor by Goldberger, Wheeler, Lillie and Rogers (1926) and found necessary for maintenance of growth and health and prevention of characteristic skin lesions in rats, and considered by the latter workers to be concerned in the prevention of human pellagra (Committee on Accessory Food Factors, 1927).

Most investigators in the past have measured vitamin B<sub>2</sub> by growth tests on rats, since they have found it difficult to produce the skin lesions with any regularity. Recent work indicates that vitamin B<sub>2</sub> in this sense covers a complex consisting of at least two factors. One constituent, flavin, has been found to have vitamin-B<sub>2</sub> activity in the sense that it is heat-stable and is responsible for some of the growth-promoting properties displayed when (for example) autoclaved yeast is fed as the supplement to purified vitamin B<sub>1</sub>. On the other hand, flavin possesses none of the characteristic anti-dermatitis actions.

At the time when last year's review was written it was as yet still uncertain whether the anti-dermatitis properties of the vitamin-B<sub>2</sub> complex resided in the flavin or in some other constituent, but it was suggested that the latter alternative seemed the more probable. The first definite evidence that this is so was published in a note by György. He reported that he had found that purified flavin, given as a supplement to purified antineuritic vitamin, was insufficient to protect rats against the lesions of the characteristic "pellagra-like dermatitis." In other words, the materials usually employed as sources of vitamin B<sub>2</sub>, such as autoclaved yeast or autoclaved marmite, contained both flavin, which exerts a growth-promoting action, and some additional factor responsible for preventing the pellagra-like lesions in the rat. As this review goes to press confirmation is at hand from Elvehjem & Koehn that flavin does not possess anti-dermatitis activity. This observation of Elvehjem & Koehn is not based, like György's work, on rats, but was carried out on chickens. Evidence of the composite nature of the supplement provided by the autoclaved yeast, so often used in work on rats, is furnished also by Chick & Copping, who find that flavin alone does not restore growth unless it is supplemented

by another fraction, which in its properties appears to resemble the "Y" factor previously described by Chick, Copping & Roscoe.

The question of nomenclature presents some difficulty. György provisionally suggested retaining the name "B<sub>2</sub>" for flavin, and calling the "rat-pellagra factor," B<sub>6</sub>. Elvehjem & Koehn, on the other hand, emphasizing the past associations of the term "B<sub>2</sub>" with the prevention of dermatitis, prefer to retain the term B<sub>2</sub> for the anti-pellagra factor and suggest reclassifying the flavins.

#### FLAVIN<sup>2</sup>

*Biological activity of flavin.*—Confirmation of the growth-promoting activity of flavin was convincingly given by György, Kuhn & Wagner-Jauregg (2), who found that it still retained its properties after it had been repurified through the silver and thallium salts, converted into the chloroform-soluble tetra-acetyl derivative, and then regenerated by saponification.

Booher has also confirmed the fact that the "vitamin-G growth-promoting activity" of whey is associated, to a partial extent at least, with its "water-soluble, yellow, fluorescent pigment." Among others who have confirmed a biological activity for flavin may be mentioned Karrer & Euler and their coworkers (Karrer & Euler; Euler *et al.*), Chick & Copping, and Guha & Biswas.

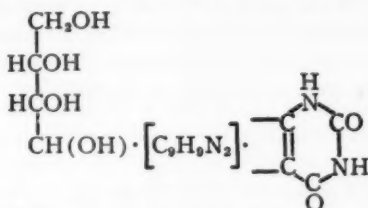
*Chemical structure of flavin pigments.*—The formula C<sub>17</sub>H<sub>20</sub>N<sub>4</sub>O<sub>6</sub> for lactoflavin was confirmed by Kuhn, Rudy & Wagner-Jauregg, after they had repurified it through the thallium and silver salts [see also Ellinger & Koschara (1)]. As Kuhn and his coworkers pointed out, lactoflavin appears to contain two of its four nitrogen atoms combined as a -NH·CO·NH- group in a ring system, while the other two are present as tertiary nitrogen, associated with the double bonds. It is the latter which seems to determine the colour. Certain of the oxidation-reduction properties of the molecule, on the other hand, are thought to be related to its sugar-like side chain [Kuhn & Wagner-Jauregg (2)].

The structure on the opposite page was provisionally suggested (Kuhn, Rudy & Wagner-Jauregg).

The later detailed chemical work on the structure of the flavins has rested largely on investigations with lumiflavin, C<sub>19</sub>H<sub>14</sub>N<sub>4</sub>O<sub>2</sub>, a chloroform-soluble, biologically inactive cleavage product which is

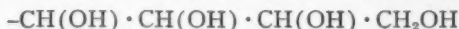
<sup>2</sup> Cf. also this volume, pp. 17, 32, 490. (EDITOR.)

formed from flavin when it is exposed to light in alkaline solution [Kuhn & Wagner-Jauregg (1), Kuhn, Rudy & Wagner-Jauregg; György, Kuhn & Wagner-Jauregg (3); Kuhn & Rudy (1, 2)].



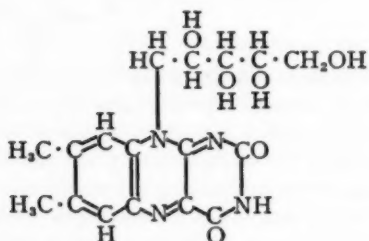
*Artificial synthesis of flavin-like substances.*—Both Stern and Kuhn and their coworkers have been able to prepare, artificially, compounds having properties resembling those of the flavins or their cleavage products. By methylation of various alloxazins, Stern & Holiday (1) succeeded in obtaining a series of homologues which had the same elementary analysis as the photolysis products of the natural flavins (e.g., "photo-hepatoflavin"), the same yellow colour, and the same green fluorescence [Stern & Holiday (1); Stern, Holiday & Stern]. In view of their uniform behaviour, Stern & Holiday (2) propose to call the whole group of these 9-alkylated alloxazins, the "photoflavins," whether prepared artificially or by photolysis from the naturally occurring native flavins.

Similarly, a mono-methyl derivative, and later a di-methyl derivative of alloxazin, were synthesised by Kuhn & Weygund (1, 2) and again shown to have properties resembling in many ways those of the flavin-cleavage products. This led the way to the synthesis of a trimethyl derivative, namely 6,7,9-trimethyl-isoalloxazin which was then found to have properties identical with those of lumilactoflavin (Kuhn, Reinemund & Weygund). Examining the oxidation-reduction properties of flavin-cleavage products, Kuhn & Moruzzi were able to sum up the relationship of the flavin group by saying that the parent substance,  $\text{C}_{11}\text{H}_{10}\text{N}_2$ , was a reductant; that combined with a ring system which contains a  $-\text{NH} \cdot \text{CO} \cdot \text{CH} \cdot \text{CO}-$  group it produced a colour; that the addition of a carbohydrate side chain containing a



group yielded a vitamin; and that the further addition of a protein group resulted in an enzyme (the yellow respiration ferment of Warburg).

Finally, as this is being written, the report has come to hand of the synthesis of an alloxazin derivative actually having the properties and biological activity of native lactoflavin itself [Kuhn; Kuhn & Weygund (3)]. It was prepared by acting on a suitable derivative of 1-nitro-3,4-xytol with *l*-arabinosamine and after reduction, condensing with alloxan. The empirical formula is given as  $C_{17}H_{26}N_4O_6$  and the structure, judging from the method of synthesis, as



6,7-Dimethyl-9-*l*-araboflavin

The dose for a rat was 0.015 mg., that is to say, approximately the same as with naturally occurring lactoflavin, and the optical rotation is the same. Kuhn states that he is conducting similar syntheses using ribose and xylose in place of arabinose to see if this synthetic product is identical in every respect with the natural substance.

*Classification of the flavins.*—At the time of writing, the interrelationships of the various naturally occurring flavins, the flavin-cleavage products (the lumiflavins and photoflavins), and the combined flavins (i.e., flavin plus protein, as in the "yellow pigment") are still being discussed.

György, Kuhn & Wagner-Jauregg (2) found that in egg white the ovoflavin consists of two flavin pigments, the more soluble of which appears to be identical with lactoflavin. In cows' milk the lactoflavin is mainly dialysable. In liver and yeast, on the other hand, it is in combination with protein, as flavoprotein. When the latter is boiled, free flavin is liberated.

Ellinger & Koschara (1, 2) would classify the lipochromes under the following headings:

1. United to protein, as in milk (or in Warburg's yellow pigment).
2. United to purines, as also in milk (lactoflavins-*a*, -*b* and -*c*).
3. The free pigments (lactoflavin-*d*, identical with ovoflavin and lactoflavin of Kuhn).
4. Lumiflavin (cleavage product formed on irradiation of 3).
5. The ether-soluble oxidation product of 4.

Guha & Biswas describe a new flavin from ox kidney, "renoflavin," which is biologically active. "Hepaflavin" from liver is dealt with by Karrer, Saloman & Schöpp. Stern (1) describes "maltoflavin," from malted barley, and "uroflavin," from urine (discussed also by Koschara). A flavin from the eggs of *Mysine glutinosa* resembling ovoflavin is mentioned by Euler and Hellström.

Euler, Karrer, Adler, Saloman & Schöpp say that an adequate dose of lactoflavin or hepaflavin for a rat is only 2 to 15  $\gamma$  per day whereas with ovoflavin a dose of 20  $\gamma$  has no effect. Meyerhoff had previously found ovoflavin to be without growth-promoting activity; but it is not clear how far these results may be due to a failure to provide other "B" factors in the diet.

*Processes for isolating flavin.*—The preparation of active concentrates has been described by several authors. György, Kuhn & Wagner-Jauregg (1), starting with liver, concentrated the flavin by means of successive precipitations with lead acetate, phosphotungstic acid, silver nitrate, and alcohol. From liver extract an active product was obtained by adsorption on fuller's earth followed by elution with pyridine.

Karrer, Saloman & Schöpp isolated hepaflavin from a watery extract of liver. After adsorption on fuller's earth or charcoal, and elution with pyridine, the flavin was precipitated with basic lead acetate. Decomposition of this precipitate with hydrogen sulphide resulted in the flavin being adsorbed on the lead sulphide so formed. This was eluted with hot water, and impurities were removed by precipitation from acetone and crystallisation from water. Another preparation was isolated by Karrer & Euler from liver, and was said to be active for rats in doses of 20 to 50  $\gamma$ . Recently, Karrer & Schöpp have given detailed directions for separating the flavin from egg yolk.

*Physicochemical properties of flavin.*—The natural flavins, as well as a number of their cleavage products, form reversible oxidation-reduction systems, which have been studied by a number of workers [Bierich *et al.*; Bierich & Lang; Stern (2, 3); Stern & Holiday;



Kuhn & Wagner-Jauregg (2); Kuhn & Moruzzi; Barron & Hastings].

Lactoflavin appears to be an ampholyte. Its pK values, determined by variations in fluorescence with pH, lie at 1.7 and 10.2.

Karrer & Euler find hepaflavin to be readily soluble in cold water, alcohol, or glacial acetic acid. György, Kuhn & Wagner-Jauregg (1) report that the biologically active material diffuses through cellophane or parchment. It is destroyed at room temperature by contact with 0.25 *N* NaOH for twenty-four hours, but not by 1.0 *N* H<sub>2</sub>SO<sub>4</sub>. Rather similarly, Guha & Chakravorty found that the vitamin-B<sub>2</sub> activity of ox kidney was rapidly lost by warming with alkali.

*Distribution of flavin in nature.*—Euler & Adler (1) have estimated the amount of flavin in animal tissues from measurement of the degree of fluorescence under suitable conditions. They give the following figures for the weight of flavin in one gram of fresh tissue: ox liver or kidney 10 to 20 γ, corpus luteum and adrenal 5 to 10 γ, brain and ovary 1 to 5 γ, spleen, lung, pituitary, and placenta 0.5 to 1 γ. The same authors (2) have also compared the amounts of "free" flavin with "combined" (yellow enzyme) by means of dialysis. In ox liver or kidney or in human brain, 70 to 80 per cent was in the combined state; in dried yeast, egg yolk, or egg white 90 to 100 per cent was combined, whereas in cows' milk the corresponding value was 20 to 25 per cent; combined flavin was not found in human urine.

By biological tests with rats, György, Kuhn & Wagner-Jauregg (1) found, in confirmation of other investigators, that liver and kidney were the richest sources of flavin. Heart muscle was five times more active than striated, being equal to yeast. Skeletal muscle varied considerably, veal and dark poultry being especially high. Spleen was high, whereas thymus and pancreas were low and carcinoma tissue very low. Biological tests for "vitamin B<sub>2</sub>" by Guha & Chakravorty led to rather similar results, kidney (ox or buffalo) being the most active source, while fowl liver was a good source, and fish liver poor.

*Physiological function of flavin.*—As a number of investigators have pointed out, the function of flavin, apparently, has some connection with oxidation processes in the cell. Structural peculiarities, such as the sugar-like side chain, presumably contribute to its distinctive character. Wagner-Jauregg, Rauen & Möller find a suggestive parallelism between respiratory intensity in muscle and liver, and flavin content. They consider the flavins to be natural hydrogen acceptors in the dehydrogenation processes of muscle. Wagner-Jauregg & Ruska

speak of flavins as oxygen carriers for the tissues. They have found that crude flavin solutions are decolorised by tissues, especially when substrates such as lactic or succinic acids or aldehydes are present. A coenzyme, present in the crude preparation, appears to be necessary for this reaction. At any rate, and whatever the precise details of the mechanism are, the wide distribution of so extremely negative a redox system cannot fail (as K. G. Stern has said) to be of physiological significance.

The yellow enzyme (the protein-flavin complex) is considered by Warburg & Christian to be concerned not only with the transfer of oxygen but also to be an enzyme for respiration in the absence of oxygen.

A negative finding, by Green & Dixon, is that lactoflavin plays no part in the oxygen uptake of xanthin oxidase.

#### PELLAGRA AND THE PELLAGRA-PREVENTIVE FACTOR

*Incidence of pellagra in human beings.*—Following on the disclosure by Thaysen of the unsuspected prevalence of pellagra in institutions for the insane in Denmark,<sup>3</sup> further searches by Bredmose, by Hofman-Bang, and by Teglbjaerg and others have revealed numerous additional cases. These it has been pointed out may be caused either by unsuitable food (for example the "sloppy diet" so often given in such institutions) or often, indirectly, by faulty absorption (a result of protracted gastro-intestinal disturbance). This second form of pellagra, or "secondary pellagra" (Strauss), may be well compared with the secondary beri-beri, already referred to on p. 332.

It is reported that pellagra is very prevalent among the Jews in Constantinople. It is attributed to the use of unleavened bread; thirty such cases are described by Sinai.

According to the compilation of Stannus & Gibson, only 131 cases have so far been described in England; the vast majority were in asylums, mostly in females. It is a tribute to recent progress to realise that, in the past, such patients have nearly always succumbed to the disease.

*The pellagra-preventive factor.*—Pellagra has been successfully treated by means of vitamin B<sub>2</sub> given in the form of liver extract, in some carefully controlled experiments carried out by Smith & Ruffin

<sup>3</sup> Cf. *Ann. Rev. Biochem.*, 3, 254 (1934).

(see also Ruffin & Smith). They showed that the same preparation might be used to cure black-tongue in dogs. Somewhat similar experiences have been recorded also by Spies, who used autoclaved yeast or ventriculin, and by Bredmose and Hofman-Bang. Mellanby has suggested that the nervous lesions in pellagra may be due to deficiency not of vitamin B<sub>2</sub> but of vitamin A (see p. 359). Further therapeutic trials, therefore, such as those just alluded to, will be awaited with much interest. In this connection reference must be made, too, to the finding of Zimmerman & Burack that marked nervous lesions, with degenerative changes and other abnormalities in the spinal cord, could be produced in dogs by a diet deficient only in vitamin B<sub>2</sub>. Again, Rhoads & Miller found that they could prevent or cure symptoms of black-tongue regularly by means of vitamin B<sub>2</sub> (or some associated substance).

*Distribution of the pellagra-preventive factor.*—Wheeler & Sebrell, continuing their valuable work on the distribution of the pellagra-preventive factor, have assayed a further fifteen foodstuffs. These tests were carried out, in the first place, on black-tongue in dogs, and then confirmed, in the more important instances, by direct tests on man. A comprehensive tabulation of the distribution of the P-P factor in foods has recently been issued by Sebrell (1).

*Pellagra-like conditions in experimental animals.*—The pathology of experimental black-tongue in dogs, brought about by diets deficient in vitamin B<sub>2</sub>, has been described in detail by Lillie. The epithelial lesions and extensive changes in the central nervous system are among the more striking of the features described. "Yellow liver," a new manifestation of vitamin-B<sub>2</sub> deficiency in dogs, is described by Sebrell (2) and by Lillie & Sebrell. Whether it is a new form of black-tongue, or possibly a distinctive condition, seems for the moment uncertain. Zimmerman & Burack, feeding dogs on diets deficient in vitamin B<sub>2</sub>, have also observed a condition different from that described usually as black-tongue; but, as they point out, the degenerative lesions seen in the central nervous system were "similar to or identical with" those described in pellagra in man.

*Cataract.*—A consistent result of deficiency of "vitamin B<sub>2</sub>" in rats and other species is described further in a long series of papers on cataract by Day (1) and Day & Langston. As Day (2) points out, the cataract-preventive factor seems to run parallel with the "B<sub>2</sub> growth-promoting fraction" in various extracts. For the present, however, its identity must remain a matter for speculation.

## THE VITAMIN B COMPLEX AND ANTI-ANAEMIA FACTORS

*Vitamin B<sub>2</sub> and pernicious anaemia.*—Although the exact connection is still obscure, a number of suggestive indications are coming to hand, all pointing to the probability of some correlation between pernicious anaemia and one or other of the factors associated with "vitamin B<sub>2</sub>."

In the first place it has been found that a deficiency of vitamin B<sub>2</sub> in experimental animals may lead to a pathological picture having certain features in common with that seen clinically in pernicious anaemia. Thus Miller & Rhoads (1) have reported that dogs deprived of vitamin B<sub>2</sub> exhibited symptoms of glossitis, stomatitis, or gastrointestinal disturbance; anaemia was present in 60 per cent and there were changes in the bone marrow resembling those of pernicious anaemia or sprue. Hutter, Middleton & Steenbock have similarly observed evidence of glossitis in rats deficient in vitamin B<sub>2</sub>.

A second line of evidence is that there are clinical analogies to be found between certain of the symptoms seen in clinical pellagra and those of pernicious anaemia. For example, many workers have demonstrated the occurrence of achlorhydria in pellagra. Thus, Mullholland & King found no free hydrochloric acid in 72 per cent of the 102 cases of pellagra which they investigated; and Helmer *et al.* noted a diminution in pepsin and rennin in the gastric mucosa in pellagra, and a complete absence in pernicious anaemia.

Yet a third argument is that there are similarities in distribution between certain anti-anaemia principles and the vitamin-B complex. It will be recalled that Strauss found that his anti-anaemia "extrinsic factor" had a distribution resembling that of vitamin B<sub>2</sub> and, like it, was heat-stable. The suggestion that vitamin B<sub>2</sub> was actually identical with Strauss's extrinsic factor was disputed by Wills, but recently Miller & Rhoads (2) have strongly questioned the validity of Wills' evidence. They have made the surprising observation that not only egg white (rich in vitamin B<sub>2</sub>) but also an acetone extract of rice polishings (containing relatively little B<sub>2</sub>) were potent sources of the extrinsic factor. Vitamin B<sub>12</sub>, in the form of the International Standard (activated acid clay), was inactive. Miller & Rhoads conclude that adequate evidence, either for the identity or for the non-identity of vitamin B<sub>2</sub> with the extrinsic factor, is still lacking. They add, however, that if "B<sub>2</sub>" is the extrinsic factor it must clearly be active in very low doses. A good deal of past work, it seems to the present

writer, is invalidated because the materials used as sources of vitamin B<sub>2</sub> were relatively little concentrated, and often of unknown activity. Further progress should be possible now that active concentrates are becoming available, and the constituents of the B complex becoming better differentiated. Experiments with purified preparations may be expected in due course to lead to important advances.

*Vitamin-B complex and macrocytic anaemias.*—A clear distinction must be drawn between such a condition as pernicious anaemia, which is due not directly to dietary errors, but to absence of an intrinsic factor, and macrocytic anaemias which may have a purely dietary origin. There is ample evidence, from the work of Wills and others, that certain types at least of macrocytic anaemias respond promptly and completely to treatment with certain materials rich in the vitamin-B complex, such as marmite. The active curative principle has not so far been satisfactorily identified with any recognised constituent of the complex.

#### VITAMINS "B<sub>4</sub>" AND "B<sub>5</sub>"

In past numbers of this review attention has been called to the difficulties which confront us in trying to recognise vitamin B<sub>4</sub> as a separate entity unrelated to vitamin B<sub>1</sub>. To summarise the position, it may be said that vitamin-B<sub>4</sub> deficiency seems to resemble a state of chronic or persistent deficiency of vitamin B<sub>1</sub>, since it can always be cured by the administration of a sufficiently large dose of vitamin B<sub>1</sub>; while vitamin B<sub>4</sub> seems to be a variation of vitamin B<sub>1</sub>, since vitamin B<sub>1</sub> cannot be obtained free from vitamin-B<sub>4</sub> activity. The published experimental work on which the case for vitamin B<sub>4</sub> is based may be outlined as follows. (a) To produce vitamin-B<sub>4</sub> deficiency the animal is first deprived of vitamin B<sub>1</sub> until it shows evidence of deficiency, and then small doses of vitamin B<sub>1</sub> are given, sufficient to cure some of the symptoms but leaving unaffected others formerly considered to be due also to lack of vitamin B<sub>1</sub>, but now held to be characteristic of vitamin-B<sub>4</sub> deficiency. (b) Should larger curative doses of vitamin B<sub>1</sub> be given the symptoms of vitamin-B<sub>4</sub> deficiency now disappear. (c) Specimens of supposedly pure crystalline vitamin B<sub>1</sub>, prepared by observers in different parts of the world, having identical properties, and giving no evidence of admixture with impurity when examined by X-ray analysis or other means, all possess their characteristic vitamin-B<sub>4</sub> activity.

Some of the difficulties of the position are frankly recognised in a recent paper by O'Brien, which deals with the methods of assay of vitamin B<sub>4</sub>. He agrees that

all B<sub>1</sub> preparations recently investigated both cure these symptoms [of vitamin-B<sub>4</sub> deficiency] and induce rises in weight. . . . For the sharp distinction of the two sets of symptoms, a basal B<sub>1</sub> preparation completely free from B<sub>4</sub> is necessary. In the absence of such a preparation, the adult curative method of assay of B<sub>4</sub> has been abandoned.

More definitely, Waterman & Ammerman, having examined the relation between the dose of crystalline vitamin B<sub>1</sub> given, and the growth response so produced, and finding the "limit of beneficial increment" to be extremely high, have now concluded:

The indications are that B<sub>4</sub>, if it exists, is present in the vitamin B<sub>1</sub> crystals, or is replaceable by massive amounts of vitamin B<sub>1</sub>. The vitamin B<sub>4</sub> hypothesis is still tenable.

## VITAMIN C

*Vitamin-C deficiency in human beings.*—Göthlin and his collaborators have continued to apply the capillary-resistance test to determine the incidence of sub-scurvy in Scandinavia. The results in different districts vary considerably, showing a good correlation with the amounts of vitamin C in the diet. In Norrland (an area north of the Arctic Circle) one "healthy" child out of every five suffers from vitamin-C undernourishment [Göthlin (1); Falk, Gedda & Göthlin]. Göthlin advocates the consumption of locally grown berries as the most practical solution. When the vitamin deficiency is made good the lowered capillary resistance returns to normal, which affords good evidence of the usefulness of this method of test. At the same time it has to be borne in mind that certain other pathological conditions, occasionally met with, apart from sub-scurvy (or scurvy), may also lead to a lowered capillary resistance. Schultzer, examining hospitalised patients in Copenhagen, found that out of 149 patients no less than forty-two had lowered capillary resistance. In twenty-five of these forty-two cases, hypovitaminosis C was the cause, whereas in the remaining seventeen various functional or systemic disorders were responsible. An alternative method of determining capillary resistance, by cupping instead of by the application of pressure, is described by Dalldorf. The method is simpler to apply, but the results cannot be

expressed so nicely on a quantitative basis. A method of greater specificity for estimating vitamin-C reserves is being developed by Harris and his coworkers. It depends on determining the "state of vitamin saturation," by means of measurements of the urinary output after the administration of large test doses of vitamin C (see Harris, Ray & Ward).

The vitamin-C content of the tissues in human beings has been found to vary considerably, e.g., from over three times the "average" value to less than one-tenth of it. The suggestion is made that 20 per cent of the subjects examined *post mortem* (in America) were in a state of latent scurvy (Yavorsky *et al.*).

The minimum daily dose of vitamin C for a human being, needed to "prevent the slightest objectively ascertainable prescorbutic alterations," would appear to be about 19 to 27 mg., assuming that certain ratios between the protective doses against scurvy and sub-scurvy for the guinea pig hold good for the human being. A child seems to need quite twice as much as an adult, per kilogram of body weight [Göthlin (1, 2)].

A result which may be of practical significance for dentistry is the finding of Fish & Harris that in the absence of vitamin C, in experimental guinea pigs, the enamel and the cementum (among other tissues) fail to develop, so that a condition simulating caries may result.

*Vitamin C and infection.*—The trend of recent experiment confirms the impression that under certain circumstances deficiency of vitamin C may cause a marked diminution in resistance to certain types of infection. Both vitamins A and C may be called "anti-infective," in the limited sense that in their absence pathological changes occur which may open the way to secondary invasion. With the B vitamins and with vitamin D, on the other hand, attempts to demonstrate an anti-infective action have not led to such convincing or clear-cut results. Some interesting tests were carried out recently by McConkey & Smith who fed tuberculous sputum to guinea pigs suffering from hypovitaminosis C and to animals on a normal diet. Ulcerative intestinal tuberculosis developed in the deficient animals and not in the controls.

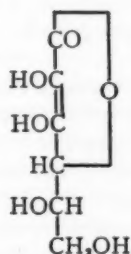
*Vitamin C and rheumatic conditions.*—Results which may possibly prove of great clinical significance are described by Rinehart and his coworkers. By combining a state of chronic scurvy in guinea pigs with a super-imposed infection, a picture was produced which had striking pathological similarities to rheumatic fever and rheumatoid



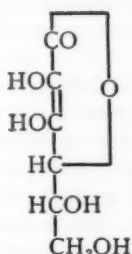
arthritis. The suggestion is made that a sub-clinical degree of scurvy may constitute the rheumatic tendency, in which the added factor of infection causes the development of rheumatic fever and, possibly, the closely allied condition of rheumatoid arthritis. The existence of a dietetic factor, long suspected in the aetiology of rheumatic fever, would certainly seem to lend added support to Rinehart's suggestions.

*Processes for synthesising vitamin C.*—Further details of technique for obtaining high yields of synthetic vitamin C are published by Reichstein *et al.* (Reichstein & Grüssner; Reichstein, Grüssner & Oppenauer). Micheel & Kraft have also described alternative reactions for the synthesis. Haworth & Hirst have discussed the chemistry of the intermediate products, and the biological activity of the synthetic product has been confirmed by Haworth, Hirst & Zilva.

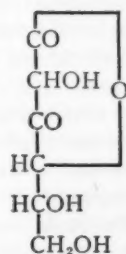
*Relationship of vitamin C and its homologues.*—"Isovitamin C" or "isoascorbic acid" (*d*-erythro-3-ketohexonic acid), a substance differing from vitamin C only in the steric arrangement of the groups on the fifth carbon atom, has been prepared and found to possess one-twentieth to one-fiftieth the anti-scorbutic activity of ascorbic acid [Maurer & Schiedt; Dalmer & Moll; Demole (1)]. *d*-Ascorbic acid is inactive at forty times the dose of the active or *l*-form. Various other allied substances and derivatives are inactive [Demole (2)].



*l*-Ascorbic acid  
Vitamin C



*d*-Ascorbic acid



*d*-Erythro-3-ketohexonic acid.  
"Isovitamin C"

*Chemical properties of vitamin C.*—"Reductic acid," a substance which is formed by the decomposition of carbohydrates and has some resemblances to vitamin C in its reducing power, has been described by Reichstein & Oppenauer.

The reversibility of the first oxidation of ascorbic acid has been confirmed [Johnson (1)]. A colour reaction depending on the formation of a vitamin-Fe complex has been discovered (Szent-Györgyi). The osazone derived from vitamin C, or "vitamosazone," has been shown to have uses for purposes of identification (Kotake & Nishigaki). The reducing potentials of vitamin C have been further examined by Fruton, who has shown that measurements with electrodes may lead to anomalous results, whereas (in agreement with the earlier results of Green), photometric studies from the colour changes of indicators furnish reproducible values for a definable reversible redox system.

*Standardisation of vitamin C.*—Harris & Ray (2) criticised the use of lemon juice as the International Standard, since they found that specimens might vary appreciably in potency. Determining the average amount of ascorbic acid in various specimens of lemon juice the relation was reached that 1 milligram of ascorbic acid contained 21 International Units of vitamin C. This is rather divergent from the value 7.4 put forward by Key & Morgan, but it agrees with the round value later accepted by the International Conference (viz. 20 I.U. per mg.). For the International Unit, 0.05 mg. of *l*-ascorbic acid now replaces 0.1 cc. of lemon juice. This relationship between ascorbic acid (the new standard) and lemon juice (the old) is in good conformity also with the later determinations of Bacharach *et al.*, who found the mean concentration of ascorbic acid in lemon juice to be 0.64 mg. per cc.

*Assay of vitamin C.*—Bacharach *et al.* agree with Harris & Ray in considering the titration method with 2,6-dichlorophenolindophenol the most specific chemical test so far available for the vitamin. An alternative method depending on the decoloration of methylene blue in the presence of light and ascorbic acid has been suggested [Viale; Martin & Bonsignore (1)]. The dyestuff "prune" may also be used in place of the 2,6-dichlorophenolindophenol, but the method is then said to be less straightforward. For removing interfering substances (cystein, ergothionene) which may also reduce the indophenol, although much more slowly than ascorbic acid, a process involving precipitation with mercuric acetate and subsequent reduction with hydrogen sulphide has been described [Emmerie; Emmerie & Eekelen; Eekelen *et al.* (2); Eekelen, Emmerie & Wolff]. The occurrence of unidentified interfering substances in germinating peas is alluded to also by Johnson (2).

Robertson has successfully used the intensity of absorption at 265 m $\mu$  for detecting and estimating vitamin C. The "irrelevant absorption," i.e., that caused by substances other than the vitamin, can be allowed for in an ingenious manner by measuring the absorption before and after destruction of the vitamin by aëration.

*Distribution of vitamin C in animal sources.*—The introduction of a method for estimating vitamin C by titration has greatly stimulated investigations on its distribution in the animal body and of the various factors affecting the latter. During the year under review about fifty papers concerned with these topics have come under the reviewer's notice. Space will not allow of an adequate citation of many interesting points raised, but it is possible to sum up some of the principal findings by saying that biological tests have now fully confirmed the indications of high concentrations of vitamin C in the following tissues: suprarenal medulla [Harris & Ray (1)], the eye-lens and the aqueous humour (Birch & Dann; Kawachi), and the vitreous humour (Harris & Robertson). (The vitamin C content of the lens is said to be lowered in cataract: see Müller *et al.*; Euler & Martius). The pituitary gland is also a highly active source, and has been studied in different states of human nutrition by means of the silver-staining method (Gough). The latter procedure has also been applied by several investigators for examining the distribution of the vitamin histologically, e.g., in the adrenal gland (Bourne).

Harris, Ray & Ward have made a quantitative study of the concentration of vitamin C in human urine and found it to vary in a peculiar manner, depending both upon the past diet (i.e., the vitamin reserves) as well as on the current intake. The average daily output by normal human subjects exceeds the reputed minimum daily dose of the vitamin. Somewhat similar results have been reported by Eekelen *et al.*, and by Johnson & Zilva (cf. Hess & Benjamin). Eekelen and his coworkers (1) have also investigated the occurrence of vitamin C in the various body fluids; in the blood it appears to occur in the reversibly oxidised form. Yavorsky, Almaden & King have made a detailed study of the distribution of vitamin C in human tissues; the results, in general, correspond with those for the rat.

*Distribution of vitamin C in vegetable tissues.*—Here the avalanche of papers is even greater, reaching a total of about one hundred. A significant fraction of these consists of the comprehensive surveys recently carried out in the U.S.S.R. (see Ivanov). In the U.S.A. detailed investigations have been made into the keeping properties of

frozen orange juice, as influenced for example by de-aëration, gas-preservation, addition of preservatives, pasteurisation, etc. (Joslyn & Marsh; Buskirk *et al.*). An important commercial development may be foreshadowed by the finding that orange juice can be kept without loss of vitamin C if it is suitably gassed with carbon dioxide (see Morgan *et al.*); possibly in the future it will be found as agreeable and advantageous to have orange juice transported in bulk in place of in the form of the separate fruit. For the preservation of the vitamin in canned tomato it is likewise necessary to remove dissolved oxygen (Kohman *et al.*).

Gerstenberger and his collaborators found that a preparation of spray-dried orange juice powder retained its activity for over fifteen months. It was tested on guinea pigs and also in one case of human scurvy, and then, when mixed with "acid protein milk," successfully cured six babies with scurvy.

Sansome & Zilva have found that polyploidy in tomatoes is related to the vitamin-C content; this result is in contradiction to the conclusions of Key, but it recalls similar observations by Crane & Zilva for apples.

By biological and chemical methods Harris & Ray (2) found the average ascorbic acid content of orange juice to be 0.6 to 0.7 mg. per cc., and lemon juice 0.47; Bacharach *et al.* by chemical methods find 0.5 and 0.6, respectively, and for tangerines, 0.37.

Zilva finds that apples contain a thermolabile enzyme, which, under aërobic conditions, may cause the rapid destruction of the vitamin.

*Origin of vitamin C in nature.*—By means of chemical tests, the synthesis of vitamin C has been followed in the chick embryo [Ray (1); Martin & Bonsignore (2)] and in the germinating seed [Ray (2)]. For the latter process mannose seemed to serve as the most effective precursor of the vitamin, since, of various hexoses added to the culture medium, it led to the largest formation of the vitamin. Disaccharides gave low results, and other carbohydrates were quite negative. This observation, of the pre-eminence of mannose as precursor of vitamin C, gains added significance from the finding of Guha & Ghosh that when certain animal tissues (liver, kidney, or spleen) were incubated with sugar solutions, mannose alone, among those tested, gave rise to a large production of vitamin C.

Harde & Wolff found considerable amounts of ascorbic acid in the walls of the small intestine of the mouse. They suggest therefore

that the site of the synthesis of the vitamin may be in this organ. The concentrations in the liver, stomach, colon, and cecum were considerably less. Independently, Hopkins has drawn attention to the "possible functions of the small intestine in the metabolism of ascorbic acid." The high concentration of the vitamin in the walls of the small intestine of the rat, he found, was maintained even when there was none in the diet, and actually rose still higher when the animal was starved. With a diet containing protein, but free of carbohydrate, the amount of vitamin C was maintained in the gut, but it fell in the liver. Whereas a diet rich in protein caused an increase in the vitamin content of the gut, carbohydrate caused a rise in the liver.

The presence of a high concentration of vitamin C in the adrenal glands raised the possibility that these organs might be the site of the synthesis in the body, but tests with adrenalectomised rats [Harris & Ray (1)] and dogs (Vars & Pfiffner) on a vitamin-C-free diet have proved that the vitamin can be formed as readily in their absence.

Eekelen & Kooy have found that when rats are fatigued by long exercise on a treadmill the ascorbic acid in the suprarenals and in the liver falls to a very low level. This seems of interest in view of the consideration that one of the first symptoms of scurvy, as of Addison's disease, is a high degree of fatigue.

The claim has been made that vitamin C can be synthesised by the young infant (Rohmer *et al.*), but the only evidence for the theory was that it continued to be excreted (for a period of forty-eight hours) when none was given in the diet. As Harris, Ray & Ward have shown, the explanation is that it is the body's reserves which are being excreted under these conditions.

*Physiology of vitamin-C action.*—A number of investigators have found that vitamin C serves to activate or to inhibit various enzyme systems. Since, however, a similar action can often be demonstrated for certain other reducing agents, e.g., for sulphydryl groups, it remains doubtful how far the effect is specific and not merely the result of a strong reducing potential or the ability to combine with oxygen. The activation of arginase by ascorbic acid plus copper has been studied by Edlbacher & Leuthardt, and of arginase by ascorbic acid plus iron by Purr (1) (cf. Klein & Ziese; Karrer & Zehender); the activation of papain by ascorbic acid plus iron by Maschmann & Hellmert (1, 2) (cf. Bersin; Hellerman & Perkins); the activation of cathepsin by ascorbic acid plus iron by Euler, Karrer & Zehender; the inhibition of urease by ascorbic acid plus copper by Edlbacher & Leu-

thardt; and the influence of ascorbic acid on plant and animal amylases by Purr (2).

The ability of ascorbic acid to protect adrenalin against oxidation, either spontaneous or through the medium of the enzyme tyrosinase (Abderhalden), is no doubt similarly due to its power of combining with oxygen. Karrer & Bendas have pointed out that ascorbic acid is also able to reduce ammonium nitrite or potassium nitrite under certain circumstances, and they ask whether it may not be concerned in processes of assimilation of nitrogen compounds. Quastel & Wheatley have found that ascorbic acid affects also the oxidation of fatty acids in the liver; in the presence of added ascorbic acid, slices of liver maintained their respiration at a higher level for a longer time, with an increased production of acetoacetic acid.

*Vitamin C and cell activity.*—A theory has been advanced by Fish & Harris to account for the action of vitamin C on dental structures. The vitamin is considered to be needed primarily for promoting the functional activity of the formative cells, i.e., odontoblasts, ameloblasts, osteoblasts, etc. The various structural abnormalities seen in vitamin-C deficiency are all found to be but the physiological sequelae of this primary mode of action of the vitamin. Sub-scurvy (or chronic scurvy) results in partial degeneration of these cells whereas acute scurvy causes their complete atrophy. This consideration, as Fish & Harris show, enables one to account for the surprising changes in the appearance of the tooth in sub-scurvy as compared with full scurvy. One is able to abandon the "pulp-bone" theory of earlier writers, according to which it appeared that with partial deficiency of vitamin C a type of neoplasm, the "pulp bone," made its appearance, and that the odontoblasts changed their function and reverted to osteoblasts. In rejecting this view, Fish & Harris show that the true explanation is that the "pulp bone" seen in chronic sub-scurvy is the natural result of a partial degeneration of the odontoblasts; it constitutes, in fact, a type of scar tissue. In full, or acute scurvy, on the other hand, the more complete degeneration of the cells is responsible for a very different and less striking picture. These results were arrived at largely by a new technique, in which the effects of a series of successive deficient and normal diets were studied in turn on one and the same tooth, as seen in a single longitudinal section. The tooth, in the case of the guinea pig, is of persistent growth, so that each of the varying portions seen along the length of the tooth corresponds with the various changes in the diets throughout the period of the experiment.

## VITAMIN A

*Vitamin A and disorders of the skin.*—Two or three years ago the suggestion that the local infections, which are so prominent a feature in vitamin-A deficiency, could be attributed to the xerosis of membrane tissues was a novel idea, not generally to be credited, or indeed still remaining unknown to the majority of workers, as reference to the standard textbooks or the contemporary journals will show. To-day this explanation has become almost universally accepted, but in turn the newer finding, that vitamin-A deficiency may also take the form of disorders in the epidermis, now needs to be familiarised.

It is more particularly in the earlier stages of the deficiency, or in a state of chronic hypovitaminosis (as distinct from avitaminosis), that the skin changes may be looked for. One of the earliest papers on the question is by Loewenthal (see also Mitchell, Owen & Loewenthal), from Uganda. From his observations in gaols in East Africa he pointed out that the first visible change in vitamin-A deficiency appears to be a dryness of the skin with a papular eruption, occurring in most parts of the body except on the face. The nucleus of the trouble appeared to be an altered pilosebaceous follicle. This peculiar kind of dermatosis is seen before xerophthalmia makes its appearance: thus among eighty-one prisoners segregated for examination, seventy-four had the dermatosis and only forty-five had xerophthalmia. The dermatosis is healed by cod-liver oil in as short a time as one to nine weeks, whereas the xerophthalmia takes longer to cure (Loewenthal). Lesions of the same kind were observed again a little later in China by Pillat, and afterwards by Frazier & Hu, the characteristic feature being the keratinised papules at the site of the hair follicles in the limbs and the trunk. More recently still Nicholls (1, 2) has pointed to the extraordinarily wide incidence of a similar condition in Ceylon, in schools, gaols, asylums, and hospitals. Nicholls applies the term "phrynoderma" to this condition, after its native name, "toad skin." He finds it to be frequently associated with another clinical picture which he names "sore mouth." These same lesions of the skin have been reported since in Uganda by Wright and one characteristic case has been seen by Goodwin in London. Now that the picture has been described it seems probable that it may be recognised as of common occurrence in many parts of the world.

MacKay, having first critically reviewed the clinical literature (1) and concluded that the earliest effects of vitamin-A deficiency



could probably be detected in the skin, undertook a large scale test on infants to determine the effects of prophylactic treatment with vitamin A [MacKay (2)].

The results taken as a whole indicate a perceptible increase in the resistance to minor infections of the skin in those infants given the extra vitamin A: the incidence of minor infections of the skin (napkin rash, intertigo, external otitis, septic spots, dribbling rash, boils, and whitlow) was 52 per cent in the control group, falling to 27 per cent in the group of infants receiving extra doses of vitamin A. It is obvious, of course, that only a fraction of these minor skin infections can be attributed to lack of vitamin A, since the result was not a "hundred-per-cent" protection such as would have followed, for example, with adequate prophylactic measures against, say, scurvy, rickets, and xerophthalmia with vitamins C, D, or A, respectively. MacKay's general conclusion is that the lowered resistance to these minor infections of the skin precedes any of the more obvious changes in the skin; and she believes that infants fed on dried milk should have their dietary supplemented with vitamin A as a routine measure.

*Vitamin A and infections.*—In the work of MacKay just alluded to, it was found that vitamin A had no effect on the incidence of general infections. The bulk of recent work is also against the view, so widely held a few years ago, that a deficiency of the vitamin in our diet is the cause of many common infectious diseases. Sutherland has made the observation, surprising enough at first sight, that extra vitamins A and D added to the diet of children of the lower working class did not have any appreciable effect even on their growth, as well as having no influence on their susceptibility to, or resistance against, infections. The explanation of this apparent anomaly is that the diet was grossly inadequate in various other respects, and that these other defects were left uncorrected. The clear moral is that medication with vitamin concentrates should not have precedence over the provision of sufficient milk; one is forced again to the realisation that the problem is not only of vitamins A, C, or D but more often is bound up with the letters L. S. D.

*Incidence of, and means of diagnosing, hypovitaminosis A.*—In Ceylon it has been established from the statistical surveys of Nicholls (already mentioned above) that vitamin-A deficiency prevails to a scarcely credible extent (see Table I, opposite).

Nicholls points out that the patients in the asylums, and the children in the poor vernacular schools, receive no more than about 30 per

cent of their calculated minimum needs of vitamin A. Keratomalacia is so common that two-thirds of the numerous cases of blindness in children are directly due to it.

TABLE I

INCIDENCE OF VITAMIN-A DEFICIENCY ("TOAD SKIN" OR "SORE MOUTH")  
IN CEYLON

Patients	Percentage
In charity boarding schools.....	83
In poor vernacular schools.....	29
In upper-class schools.....	3
In mental asylums.....	44
In mental asylums (on special European diet).....	2

Before any marked clinical symptoms are visible a "preavitaminous" condition can often be detected from ocular disturbances. For example, O'Brien has found that 10 per cent of children admitted to hospital at Iowa City had mild hemeralopia, yielding to treatment with cod-liver oil. In Greece, "idiopathic day blindness," having a dietary origin, and curable by vitamin A is said to be endemic (Joachimoglu & Logara). Details of the technique for determining moderate degrees of vitamin-A deficiency, based on the sensitivity to light following partial dark adaptation, are described by Jeans & Zentmire. Of the 213 children whom they examined, forty-five had subnormal dark adaptation. Of these, twenty-one were treated with cod-liver oil and were then found to return to normal in an average of twelve days. No less than 21 per cent of the children investigated (lower economic classes, and physically afflicted) had some degree of vitamin-A deficiency.

The minimum daily dose of vitamin A needed by a man, woman, or child, according to the calculations of Fraps & Treichler, seems to be about 1000 "Sherman-Munsell rat units" per day.

*Vitamin-A deficiency in animals.*—A convincing proof has been given by Dunlop that diets such as are commonly prescribed for swine may lead to spontaneous avitaminosis A, marked by paralysis and incoördination.

Monkeys and guinea pigs on diets deficient in vitamin A have generally been found to develop symptoms other than xerophthalmia, but this "classical" symptom has recently been shown to occur occasionally in these species also (Hetler). Range cattle may develop

the avitaminosis, under natural conditions during the dry season (Hart & Guilbert); it has also been produced experimentally (Guilbert & Hart). In horses a keratoplastic reaction in hoof formation appears to be due to vitamin-A deficiency (Klemola). Cockroaches need vitamin A, as well as the vitamin-B complex, but not vitamin C (Moskalenko).

*Vitamin-A and the nervous system.*—The nervous lesions in vitamin-A deficiency have been much stressed of late, especially in the work of E. Mellanby. This opportunity may be taken to sum up the present position, especially since reviews of past work are not readily available elsewhere. The earliest observations seem to be those of Hart & McCollum who, as long ago as 1914, found that pigs on certain experimental basal diets developed striking symptoms of incoördination, which could readily be cured by the provision of butter fat. In continuation of these observations, Hart, Miller & McCollum pointed out, in 1916, that marked degenerative changes occurred in the nervous system of these animals and that they were to be attributed to a deficiency of "fat-soluble A." A peculiar staggering gait was the most characteristic symptom associated with the lesions. This symptom occurred likewise in dogs, as a result of deprivation of vitamin A [Steenbock, Nelson & Hart (1, 2)]. Mellanby, in 1926, mentioned that his experimental puppies which were being maintained on diets deficient in fat-soluble vitamins developed symptoms associated with nerve lesions, which he attributed to the toxic influence of large amounts of cereal in the diet. Hughes and his coworkers, in 1928 and 1929, published the records of their extended observations on the effect of deficiency of vitamin A in pigs, in chicks, and in cows, in giving rise to symptoms of incoördination and spasms. As they showed, these could be correlated with a degeneration of the nerve bundles in portions of the spinal cord. The morbid histology of the disease as it affects dogs was discussed further by Mellanby in 1930–1931. In his latest work he has made a close study of the degenerative changes in the first branch of the trigeminal nerve and its cells of origin, in rabbits, dogs, and rats suffering from vitamin-A deficiency. Mellanby supposes that the xerophthalmia in vitamin-A deficiency has to do with degeneration of the nerve supply to the cornea; and, more generally, he suggests that the hyperplasia or metaplasia of the various membrane tissues throughout the body may be related to changes in their afferent nerve supply. Along the same lines, M. Mellanby & King suggest that a loss of neurotrophic control may

be responsible for the periodontal metaplasia seen in vitamin-A deficiency, and hence a cause of pyorrhoea and, possibly, even of dental caries.

Mellanby has particularly sought to find clinical parallels for the various lesions to be seen in the experimental animal suffering from vitamin-A deficiency. Apart from the dental aspects just touched on, and the proposed use of vitamin A for the treatment and prophylaxis of septicaemias and infectious diseases (see p. 356), he suggests that vitamin-A deficiency may also be the cause of the degenerative changes in the nerves in all of the following diseases: beri-beri, pellagra, disseminated sclerosis, pernicious anaemia, lathyrism, convulsive ergotism. These ideas have been developed at length in Mellanby's recently published book (5) which is based on his Croonian lectures.

Important claims are therein made for vitamin-A therapy in disseminated sclerosis. Thirteen patients were treated with diets rich in vitamin A and all are said to have greatly improved, and "so far as can be seen at present remain improved." Mellanby remarks:

Whatever may be said about remissions so commonly seen in this disease, it is well known that the general tendency is for patients suffering from disseminated sclerosis to become gradually worse, and this has certainly not happened in the 13 cases since they were first examined. . . .

In one of the two cases of pernicious anaemia examined, large reserves of vitamin A were found, *post mortem*, in the liver, and Mellanby therefore suggests that the vitamin A

although present in the liver of these patients cannot, for some unknown reason, carry out its ordinary work of controlling nerve function and structure.

On the other hand Zimmerman & Burack (see p. 344) have found that simple deficiency of vitamin B<sub>2</sub> without any deprivation of A suffices to produce in experimental animals degenerative lesions said to be identical with those seen in clinical pellagra; recent direct tests on human beings have likewise proved that the pellagra-preventive factor is associated with foods and extracts rich in vitamin B<sub>2</sub> and not vitamin A (e.g., Wheeler & Sebrell, see p. 344). It has also been found experimentally that a deficiency of vitamin B<sub>1</sub> may similarly produce experimental lesions in the central nervous system (Prickett; see also Gildea *et al.*). Clinically, vitamin B<sub>1</sub> without A is widely and successfully used in the treatment of beri-beri (e.g., as Jansen's activated clay, yeast extract, etc.). It has also been argued that the lesions

in the spinal cord produced experimentally in vitamin-A deficiency differ histologically from those seen clinically in sub-acute combined degeneration [*Lancet*, 1, 197 (1934)]. A further difficulty is as follows: that if chronic vitamin-A deficiency is the cause of the nervous lesions in the list of diseases mentioned above, one might expect to see evidence of its better-known symptoms, such as local infections and xerophthalmia.

Clinical tests with vitamin-A concentrates should do much to settle these questions.

*The vitamin-A cycle in the eye.*—The fascinating processes by which the visual purple and other pigments in the eye are elaborated from vitamin A and then again degraded have been studied by Wald. In outline, vitamin A, brought to the retina by the circulation, combines with protein to give visual purple; this, by a purely photochemical reaction, is then converted into the "visual yellow," which afterwards breaks up again, yielding vitamin A and other colourless products. Wald describes also a new carotenoid pigment, "retinene," derived as an artifact from the visual yellow. It can be extracted in quantity from the retinas of dark-adapted but not of light-adapted animals, where it is replaced instead by newly formed vitamin A.

*Absorption, storage, and transmission of vitamin A.*—Baumann, Riising & Steenbock (1) found that although the amount of vitamin A stored in the livers of rats went parallel with that in their diet, yet only 10 to 20 per cent of the total amount of vitamin ingested was recovered from the organ. A considerable quantity must have been destroyed, day by day, in the body.

Dann (2) has confirmed the fact that, even when large doses of vitamin A are fed to a lactating female, the amount transmitted to her offspring remains limited. The percentage of fat in the diet had a perceptible, if scarcely important, effect on the result.

In renal diseases in human beings the amount of vitamin A found in the liver *post mortem* is persistently low (Moore). The data are striking enough even if the explanation still remains uncertain.

*Chemical constitution in relation to vitamin-A activity.*—As is well known, the several isomeric forms of carotene (as also of a number of related compounds) may differ in the presence of either an  $\alpha$ - or a  $\beta$ -ionone ring, and it seems established (see Kuhn) that it is the presence of a  $\beta$ -ionone ring in combination with four conjugated double bonds which is the criterion of vitamin-A activity. The very logical suggestion has now been made that  $\alpha$ -carotene should hence-

forth be known as " $\beta,\alpha$ -carotene,"  $\beta$ -carotene as " $\beta,\beta$ -carotene," and  $\gamma$ -carotene as " $\beta$ -lyco- $\beta$ -carotene" (Palmer). It has been confirmed (Kuhn, *et al.*) that  $\beta$ - (= " $\beta,\beta$ -") carotene, which on scission gives two molecules of vitamin A, has twice the activity of  $\alpha$ - (= " $\beta,\alpha$ -") carotene which gives only one. The demands of theory are therefore well substantiated in this respect. The structural formulae for  $\alpha$ - and for  $\beta$ -carotene<sup>4</sup> now seem established beyond doubt (Kuhn & Brockmann; Kuhn *et al.*) and full details have been given for their separation by means of chromatographic methods (e.g., Karrer *et al.*; Strain).

The vitamin-A activity of yellow maize appears to be due, after all, not to carotene itself but to a related pigment, cryptoxanthin (Kuhn & Grundman), apparently identical with the caricaxanthin of Yamamoto & Tin (see Karrer & Schlientz) and resembling the inactive zeaxanthin, studied by Euler, Karrer & Zubrys.

*Vitamin-A standards.*—The International Conference (League of Nations, Health Organisation) has substituted pure  $\beta$ -carotene for the mixed specimen of carotene previously used. The unit is the biological activity of 0.6  $\gamma$  of this substance, equivalent to that of 1  $\gamma$  of the old material. The standard is now distributed, dissolved in coconut oil. A well-tried specimen of cod-liver oil has been chosen as a secondary standard. Determinations by the spectroscopic method (i.e., coefficient of absorption at 328  $m\mu$ ) are recognised as satisfactory for liver oils and concentrates under certain strictly defined circumstances, and the relation between the coefficient of absorption and the biological activity is given.

*Method of assay of vitamin A.*—Hilger's "vitameter A" furnishes a simple method of measuring the coefficient of absorption at 328  $m\mu$ ; it gives reproducible results provided that the saponifiable material is removed before the test is carried out (Wolff & Eekelen). Some experience in optical procedure seems advisable, however, on the part of those using such an instrument; otherwise its very simplicity may give a false sense of security.

For the antimony-trichloride test, a rapid routine method suitable for applying to animal tissues, and involving preliminary digestion with alkali, is described by Davies. It has been stated that the Carr-Price test can be improved by the addition of catechol; the colour is

<sup>4</sup> Cf. Karrer, P., and Helfenstein, A., *Ann. Rev. Biochem.*, 1, 551 (1932); Kuhn, R., *Ann. Rev. Biochem.*, 4, 479 (1935).

made more permanent by this modification; carotene does not react and can, therefore, be readily differentiated (Rosenthal & Erdélyi).

Biological methods have been discussed by numerous workers. Dyer, Key & Coward make the disconcerting observation that the use of different solvents may affect the apparent potency five- or six-fold. It is sufficient to give the supplement as one single dose (Sherman & Todhunter) or twice a week (Coward & Key), instead of daily. Sherman & Todhunter prefer to calculate their results from the area bounded by the weight curve. Results are best calculated starting from the day on which the maximum weight during the depletion period is regained and not from the day when the dosing started [Baumann, Riising & Steenbock (2)]. It has also been emphasised that weight curves are not an index of growth (Orr & Richards), and that pathological changes may be far advanced before growth ceases (Richards & Simpson); for these reasons prophylactic tests are advocated. Practical difficulties have always prevented their general adoption in the past.

The use of the cockroach as a test animal has been proposed (Moskalenko, cf. page 333).

*Distribution of vitamin A and carotene in micro-organisms.*—That certain micro-organisms synthesise carotene has long been suspected and is now definitely proved. There is no evidence that vitamin A is formed under the same conditions (Ingraham & Baumann; Baumann, Steenbock, Ingraham & Fred; Chargaff).

From biological and colorimetric tests, Drummond & Gunther have concluded that vitamin A is present in phytoplankton oil but probably absent from zooplankton oil—the reverse of what has been found for vitamin D.

*Distribution of vitamin A in the animal kingdom.*—The observations made by Dann, with experimental animals, that relatively little vitamin A can be transferred from the mother to the young by the placenta or in the milk, is confirmed by the finding that the new-born or suckling infant has very low reserves of the vitamin (Debré & Busson). Drigalski has made further detailed studies on the distribution of vitamin A in the human body. It appears that the maximum level of vitamin A in the serum in normal children is reached about four hours after the food is given; it may be notably delayed in idiopathic steathorrea (Chesney & McCoord).

The importance of colostrum as a source of vitamin A has been stressed: in the first day the calf may obtain from it as much vitamin



A as in twenty to fifty days' supply of the later milk [Dann (1)]. Similar findings have been given for human colostrum (Eekelen & de Haas).

The potency of cows' milk varies not only with the cows' diet, and therefore seasonally (e.g., Booth, Kon, Dann & Moore; van Wijngaarden; Russell), but also apparently with the breed of the cow (Baumann, Steenbock, Beeson & Rupel).

The existence of a seasonal variation in the potency of halibut-liver oil has been confirmed; it moves inversely with the content of oil in the organ (Bills *et al.*). Other sources, particularly rich in vitamin A, examined during the year include salmon-egg oil and salmon-liver oil (Lee & Tolle), sturgeon liver (Bailey), and the pupa of the silk worm (Izume *et al.*). The activities of a large number of Indian foodstuffs and fish-liver oils have been measured by Ghosh & Guha, and Ghosh, Chakrovorty & Guha.

*Vitamin A and metabolism.*—Green (1) finds that vitamin-A deficiency, in the rat, produces a large decrease in the esterase content of the blood serum. Since a similar change occurs in infected rats kept on complete diets, or in rats inoculated with sarcoma, the precise significance of Green's observation is still uncertain. Fat metabolism does not seem to be obviously altered, in the sense that fat is still absorbed, mobilised, desaturated, and oxidised, in the absence of vitamin A [Green (2)]. On the other hand it is maintained that administration of vitamin A causes a marked rise in the serum cholesterol (Jusatz).

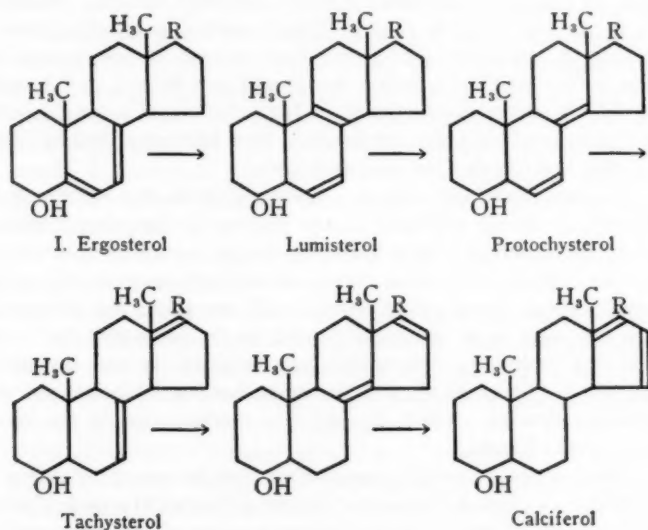
Disturbances in calcium metabolism may be caused by excess of vitamin A (Collazo & Rodriguez; Davies & Moore; Harris & Moore), or of carotene (Ishida), and a further analysis of this action will be awaited with interest.

Mellanby supposes that vitamin A works "in harmony with milk proteins, and other proteins of high biological value" in promoting the resistance of the epithelial cells, as well as in antagonism with cereals.

## VITAMIN D

*Structural formulae of ergosterol, calciferol, and intermediate sterols.*—Although the carbon "skeleton" seems no longer in doubt, the exact details of the positions of the double bonds and of the hydroxyl group in the ergosterol-ring system are still under discus-

sion. Three alternative formulae have been put forward. That of Rosenheim & King is shown in formula I. According to these investigators activation of ergosterol is the result of three intramolecular changes: (a) epimerisation of the hydroxyl group; (b) movement of the system of conjugated double bonds in three distinct stages from ring II into ring IV, involving (c) a steric change in the ring system leading from the *allocholane* to the *cholane* structure:

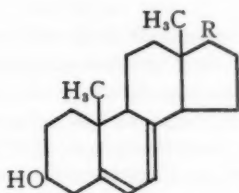


Windaus, Inhoffen & Reichel have suggested the alternative formula (II), which differs in the position of the hydroxyl group. Dunn, Heilbron *et al.* criticise both of the foregoing formulae, on the grounds that certain reactions indicate that the nuclear double bonds cannot be conjugated, and, on the basis of various experimental results, prefer formula III.

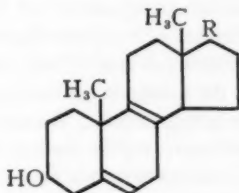
Cook & Haslewood have likewise described experimental results which are difficult to reconcile with Windaus's formula.

Danielli & Adam, from their measurements of surface pressures and surface potentials of monomolecular films of ergosterol and its irradiation products conclude that a considerable change in the angle

of the tilt of the hydroxyl group to the ring system occurs during irradiation. Their results prove that the positions of the double bonds change during irradiation and appear to furnish evidence in favour of position 3 for the hydroxyl group.



II. Ergosterol (Windaus's formula)



III. Ergosterol (Heilbron's formula)

*Are there several forms of vitamin D?*—The suggestions made in the past that vitamin D exists in more than one form have generally been based on certain discrepancies observed in the responses of different species. That is to say, a chicken, or an infant, has been observed to respond more readily to vitamin D when given in the form, say, of cod-liver oil or irradiated milk than when the same number of "rat units" of the vitamin were given in the form, say, of irradiated ergosterol. In the present writer's opinion, in some of this past work, insufficient attention may have been paid to effects caused by the unequal absorption and utilisation of the vitamin, as well as to the possibility of secondary rachitic influences of other major constituents of the diet. A rather different line of evidence was that given by Kon & Booth (1), who showed that the antirachitic activity of autumn or winter butter could not be quantitatively recovered from its non-saponifiable residue. In control tests with cod-liver oil or with irradiated ergosterol, on the contrary, a quantitative recovery was possible. This suggested that the vitamin existed in two forms, a "labile" form, disappearing when the butter was saponified, as well as the more stable or "recoverable" form. In a later paper Kon & Booth (2) find that the labile form amounts to as much as 75 per cent of the total vitamin D in the butter. Similar results are quoted by Rygh for the vitamin D in hay, in cows' liver, and in butter fat. But in a further paper Kon & Booth (3) frankly admit that their results could be more simply explained by the assumption that the fat itself in the original butter possesses some antirachitic properties.

The existence of discrepancies between the responses of different species is confirmed further by the finding that infants respond better to the vitamin D in irradiated milk or viosterol than to cod-liver oil (Drake, Tisdall & Brown); or that chicks respond better to cod-liver oil (Russell *et al.*; Dols) or to irradiated cholesterol (Waddell) than to irradiated ergosterol.

Ender, who prepared a concentrate of vitamin D from fish-liver oil, concluded that it had different chemical and physical properties from the synthetic material prepared by irradiating ergosterol. On the other hand, Dalmer, Werder & Moll find that such comparisons are inconclusive unless care is taken to remove all vitamin A. Taking such precautions they find that the natural vitamin D from fish-liver oil has all the biological properties of that in irradiated ergosterol. The ratio between toxic overdose and the therapeutic dose was also the same.

*Assay of vitamin D.*—A result reached incidentally by Bruce & Callow (1), in the course of their investigation into the "anticalcifying action" of cereals, deserves to be noted by those concerned with the estimation of vitamin D; namely, that the combined effect of altering the mineral composition of the diet and its vitamin-D content is more nearly proportional to the product of these two factors acting independently than to their sum.

Different samples of yellow corn as used in the Steenbock diet may differ in their apparent antirachitic action and in their calcium or phosphorus content (Harris & Bunker), and to correct for such fluctuations it is recommended that the Ca/P ratio of the diet be correspondingly adjusted (Davies).

*Distribution of vitamin D in nature.*—The amount of vitamin D in halibut-liver oil, like that of vitamin A, varies seasonally, inversely with the amount of oil in the organ (Bills *et al.*). Burbot-liver oil has an activity equal to or greater than that of the cod [Branion (1)] and tunny-liver oil is another very active source (Schmidt-Nielsen & Schmidt-Nielsen). Further work has been published concerning the origin of the vitamin D of cod-liver oil, and it has been shown that dried copepod "contains sufficient vitamin D to make this constituent of the zooplankton a good source in the food of the cod" (Copping; Drummond & Gunther).

According to Metz & Coppens (see also Coppens & Metz) the lung in mammals contains an enzyme which continuously destroys vitamin D, and this accounts for the large quantity of vitamin D in

the liver of fish (which have no lungs). Light and his coworkers have found that vitamin D fed to cows is absorbed in the blood stream but largely destroyed, and some is re-excreted. Only about 2 to 3 per cent of it appears in the milk.

The effects of sunshine and of the diet of the hen upon the vitamin-D value of the eggs have been discussed by Branion (2), by Bisbey and his collaborators, and others. Bethke, Record & Wilder find that a given amount of vitamin D, fed to hens, is about twice as effective in raising the vitamin-D value of the egg yolk when administered as cod-liver oil concentrate than when given as irradiated ergosterol.

Cacao shell is described as "rich in vitamin D"; it contains 28 units of vitamin D per gram (Knapp & Coward), so that about one-fifth of a gram per day would be needed by a rat for curative treatment to be fully effective.

*"The anti-calcifying principle" of cereals.*—Bruce & Callow (2) have now published a fuller account of their investigations on the "rachitogenic" or "anti-calcifying" factor in cereals ("anti-vitamin D"), described by Mellanby. In brief, their results show that the reason why cereals favour the production of rickets is that their phosphorus is contained in a non-assimilable form, namely that of inositol-hexaphosphoric acid. As Bruce & Callow point out, a clue to the secret had already been given by Steenbock, who had referred to the differences in the "biological availability" of the phosphorus in different sources, and it was already known that the phosphorus of phytin was not readily absorbed. In the authors' own words,

a series of quantitative comparisons has shown that the differences between oatmeal, maize + white flour, and maize can be completely accounted for by differences in the total phosphorus content and in the proportion of inositolhexaphosphoric acid. . . . The relative proportions of phytin-phosphorus and other phosphorus in cereals is of significance in diets used for vitamin D testing.

Administration of sodium inositolhexaphosphate by mouth to monkeys produced no specific nervous symptoms. Thus it appears that this substance is not related to the toxamins of cereals which produce nervous degeneration.

*Rickets in animals and human beings.*—The incidence of rickets, and the vitamin-D requirements of various farm animals, e.g., pigs, ewes, chicks, hens, and calves, have been discussed in numerous articles. Martin & Pierce have emphasised the importance for sheep of the phosphorus content of their diet, and Theiler has pointed out that both bovines and ovines develop rickets (or osteomalacia) in presence

of sufficient solar radiation, under conditions of simple phosphorus deficiency. For chickens a full allowance of calcium phosphate is in itself insufficient to protect against rickets in the absence of vitamin D (Higgins & Sheard).

Blackberg & Knapp have described ocular changes seen in puppies fed on rachitogenic diets, which they say resemble those seen clinically in cases of marked dental decay due to a disturbed calcium-phosphorus metabolism.

It is of interest to learn that vitamin D, purchased as pure crystals, now costs only one-eighth of its price in cod-liver oil, and the daily dose can be bought for less than one-tenth of a penny (Carr).

*Vitamin D in the teeth.*—The third part of M. Mellanby's report has now appeared, and gives a full discussion of the effects of vitamin D upon dental structure and dental disease in children. The frequent association of rickets with badly formed teeth and with caries had been recognised in the second half of the nineteenth century, and Mellanby's observations may be said to develop this theme. Her arguments rest on three main theses. In the first place, she shows that while the "average" tooth is of poor structure and liable to decay, its architecture may be improved by proper attention to diet. Secondly, she has produced clinical experimental data to show that the addition of vitamin D to the diet helps to check the spread of caries. Finally, she discusses the incidence of caries in communities in different parts of the world and draws a correlation between special dietary habits and freedom from the disease. Translating her findings into terms of practical dietetics she advocates a diminution in the intake of cereals—or their "complete abolition" for the very young. Vitamin D, and with it the calcium and phosphorus which it controls, are the principal elements conducive to the formation of secondary dentine and therefore to the arrest of caries.

Similar conclusions have been reached by Drain & Boyd, who have investigated and checked the diets of over 500 dental patients, and have found that caries could be arrested by provision of sufficient "protective foods": cod-liver oil, milk, fruit, and vegetables. Boyd and his coworkers have found that a good correlation exists between the retention of calcium or phosphorus in the body (which, as is well known, vitamin D helps to maintain) and the resistance of the tooth to decay.

Similar conclusions are given in the U.S.A., both by McBeath who has carried out tests on a group of 425 boys and girls, aged eight to

fourteen years, and found vitamin D to be an important factor in the dietary control of caries, and also by Anderson and his coworkers who obtained very similar results.

It probably will not be disputed that other factors as well as vitamin D are involved, both dietary and otherwise, in promoting resistance to decay. Day & Sedwick found, as the result of their observations on 147 children on experiment and on 171 "controls," that no benefit resulted from vitamin D when the diet was already reasonably good, and that under such circumstances vitamin D was not the limiting factor. Hess, Abramson & Lewis again found that cod-liver oil might be ineffective in preventing caries from developing in children between the ages of seven and nine years, and, while in negro children early rickets was commoner than in white children, in later life caries became less common in negro children than in white children. It would certainly be a mistake to regard vitamin-D deficiency as synonymous with rickets, and many recent experiments on rats support the contention that in caries provoked by the absence of vitamin D it is the severity of the hypophosphataemia, *per se*, and not necessarily the severity of any rachitic lesions, which is the factor determining the incidence of caries (e.g., Agnew, Agnew & Tisdall).

Another dietary factor probably promoting resistance to decay is vitamin C, since Fish & Harris have shown that in its absence the production of dental enamel and of cementum may cease altogether. The importance of local oral factors is stressed by other workers. Many clinicians, for example, have found a correlation between the places in the teeth where carbohydrate food lodges persistently and where caries commences (e.g., Breese). In rats, the production of caries on a rachitogenic diet, such as that of Steenbock, is said to depend on the presence of coarsely ground particles of corn meal which may cause physical injury to the tooth (Hoppert *et al.*; Shelling & Asher; Lilley & Wiley; Rosebury *et al.*; Bibby & Sedwick). Again Koehne & Bunting have reported on the surprisingly low incidence of caries in an orphanage where no conscious effort had been made to that end. A special feature of the regimen here was that no candy was allowed and fruit and raw apples were eaten after meals, which seems to suggest the factor of "dental hygiene." To the present reviewer, at least, the dietary scale seems to have been very liberal from the start, compared with existing conditions in many parts of England, for example; but Koehne & Bunting contend that the immunity from caries could not be explained on the basis of the superior nutritional value of



the food, and they point out that at least half the calorific intake was derived from starchy food.

*Mode of action of vitamin D.*—Crimm and his colleagues, investigating the action of large doses of vitamin D on tuberculosis patients, confirm the contention of Harris that vitamin D increases the net absorption. Similarly Pugsley & Anderson find that vitamin D reduces the excessive faecal excretion of calcium brought about by thyroxin. On the other hand Taylor, Weld & Sykes dispute the statement that vitamin D increases net absorption in normal dogs. The explanation of the apparent anomaly may be, as Harris pointed out, that the characteristic action of vitamin D is most clearly demonstrated at high and low threshold values, i.e., in hypovitaminosis and in incipient hypervitaminosis.

The increased calcification of bone and the calcification of soft tissue in hypervitaminosis were attributed by Harris to the hypercalcaemia and hyperphosphataemia produced, and not to any direct action of vitamin D on the tissues in question. Rosenheim & Robison have now demonstrated that calcification may, in fact, be produced *in vitro*, in kidney, lung, or aorta, by simply increasing the calcium and phosphorus product, and that the picture resembles that seen in hypervitaminosis. Further, vitamin D added direct to bone slices has no demonstrable effect on their calcification (Robison & Rosenheim).

According to Skill & Kay, osteoporosis in rats, like rickets, is marked by a diminution in the amount of phosphoric esters in the red blood cells and in the liver, and by a diminished activity of the phosphatase in the kidney. Kay & Skill point out that beryllium rickets can be prevented by sodium glycerophosphate, which they take to be further evidence in support of the view that vitamin D influences the net absorption; when the rickets is cured a rise is seen in the amount of phosphoric esters in the liver and the red blood cells.

McGowan & Emslie suppose that vitamin D functions by "setting free nascent phosphoric acid from body tissues," thus exerting an acidotic tendency.

## VITAMIN E

*Vitamin-E concentrates.*—Reports on active fractions have recently been issued by three groups of workers: Martin, Moore, Schmidt & Bowden; Drummond, Singer & Macwalter; and Olcott & Mattill. Martin, Moore, Schmidt & Bowden make further observa-

tions on the association between vitamin-E activity and an absorption band at 294 m $\mu$ . The fraction which showed the most marked absorption was active for a female rat when given in a single dose of 16 mg. It was prepared from wheat-germ extract by removal of the saponifiable matter, followed by chromatographic absorption, and fractional distillation in a high vacuum. The process adopted by Drummond and his colleagues involved the removal of crude sterols by treatment with methyl alcohol at  $-30^{\circ}$ , and subsequent selective adsorption. It is agreed that biological activity seems to be correlated with a band at 294 m $\mu$ . The most active fraction was a pale yellow oil, the minimal dose of which was 0.1 mg. per rat per day. The relative stability of the vitamin is confirmed. It appears to have an unsaturated structure but can be hydrogenated without loss of activity. It may be a cyclic ketone with a molecular weight of about 450 and two oxygen atoms, one of which can be acetylated.

The paper by Olcott & Mattill gives an extensive summary of investigations which have covered a period of four years; various physical, chemical, and physiological properties of the vitamin are discussed. Lettuce and wheat-germ oil were the raw materials used. It is confirmed that vitamin E is destroyed by bromination and treatment with potassium permanganate, whereas it resists mild oxidation with silver nitrate, acetylation, benzoxylation, or hydrogenation.

*Occurrence of vitamin E.*—The occurrence of vitamin E in legumes is discussed by Zagami and in soy beans by Suzuki *et al.*

*Clinical applications.*—Vogt-Møller has investigated the treatment of sterility and habitual abortion in human beings by means of wheat germ or wheat-germ oil. To control his results properly he applied treatment only to those cases where medical examination of the man and the woman showed "apparently normal conditions." The results seem promising, for in twenty cases of habitual abortion which were treated, the birth of a living child was obtained in seventeen.

## VITAMIN F

The name "vitamin F" is given by Evans, Lepkovsky & Murphy to the essential unsaturated fatty acids discussed by Burr and Evans and their coworkers in earlier reports.

Three papers by Evans and associates have recently appeared, all dealing with the effects of vitamin-F deficiency on reproductive function. In the first paper it is stated that in the absence of vitamin F a

failure in reproductive function always results. It is marked by a peculiar and characteristic prolongation of the gestation period, due, apparently, to a derangement of the birth mechanism. The addition of vitamin F to the diet causes a marked improvement, but conditions are still abnormal and cannot be entirely rectified by the addition of a more generous allowance of the other known vitamins; for under such conditions the weight of the young is found to remain subnormal. The further addition of a large amount (25 per cent) of lard or butter to the diet, however, enables the young to thrive normally.

In the second paper results are recorded in which diets were used containing "saturated fatty acid as their sole source of energy." On such diets, in the absence of vitamin F, gestation was always unsuccessful. With the addition of vitamin F gestation proceeded normally, although lactation was still not normal.

In the third paper it is recorded that males also become sterile on fat-free diets: the addition of vitamin F suffices to cure or prevent the trouble. The nature of this sterility in the male is still to be investigated.

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## NUTRITION\*

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The subject of nutrition occupies a unique position in these reviews in that its territory is unusually broad and not well defined, and its basic constituents (protein, fats, carbohydrates, minerals, vitamins, etc.) are discussed independently in other sections. This situation affords the reviewer an opportunity for experimenting. In volume III one topic was reviewed extensively; this year several topics are reviewed briefly.

### ADULT NUTRITION AND LONGEVITY

The distinguishing feature of contemporary nutritional research is its devotion to problems of dietary deficiencies in the young. This is as it should be in times of relative scarcity of food and of nutritional knowledge. As the era of scarcity passes into one of abundance, signs appear of a budding interest in the less pressing problems of nutritional care of the adult and aged and of dietary excesses. The following examples illustrate investigational possibilities in this field: advanced senile cataract was developed in rats by excessive feeding of lactose (1); atherosclerosis was developed in rabbits by feeding cholesterol or egg (2, 3, 18); the number of islands of Langerhans was reduced in fish by feeding excessive amounts of starch and lard (4); the length of life of rats was increased by caloric underfeeding during growth (5), and by increasing the milk and vitamin-A fractions of an exclusive milk-whole wheat diet (39).

*Is rapid growth physiologically compatible with long life?*—Osborne & Mendel (6) thought that it was not. The reviewer (7) published a graph representing the relation between observed "normal" speed of approach to maturity (represented by growth constants) and duration of life, showing that rapid growth is associated with early death. This graph merely gave quantitative expression to an old rule that the life span is about five times the growth span. McCay & Crowell (5) presented experimental data on the mean longevity of rats which were full-fed, reaching maximum body weight in 4 months, in contrast to that of rats which were under-fed (diet com-

\* Received December 31, 1934.



plete except for energy), requiring  $1\frac{1}{2}$  to 2 years to reach maximum body weight. The under-fed animals maintained their youthfulness and attained a life span far beyond their full-fed mates. At the end of 37 months (equivalent to 112 years in humans, according to McCay & Crowell) only 1 of 36 full-fed rats survived, but 21 of 70 under-fed rats survived; the mean duration of life of the 36 full-fed rats was 509 days (equivalent to 51 years in humans) and of the 70 under-fed rats 780–870 days (80 or 90 years in humans). McCay, Ku, Woodward & Sehgal (8) found that rats fed 10 to 20 per cent cellulose in their diets outlived other rats on stock diets. This longevity was attributed solely to the slowness with which these animals reached maturity. It thus appears that the life span can be prolonged by prolonging the growth period. The nutritionists' ideal of rapid growth in the young seems incompatible with the desire for prolongation of life. May it not be possible to combine early maturing in the young by a high dietary level with retarded senescence in the old by a low dietary (caloric) level? If senescence is a physico-chemical aging of the protoplasm as a function of time then early maturing must be followed by a corresponding early death;<sup>1</sup> if senescence is largely an accumulation of metabolic products (e.g., cholesterol accumulation in arteries in case of atherosclerosis), then, within certain limits, an acceleration of growth may be compatible with a retardation of senescence when both are under proper nutritional control. The important problem thus emerges of determining the nutritional conditions which influence the rate of senescence.

*Is diabetes in the adult a disease of overnutrition?*—Diabetes, like most other diseases, is the resultant of heredity and environment. The hereditary aspect of diabetes is pathetically clear in diabetic children. But how should one explain the rising tide of diabetes in adults? It is not original to say that this may be due to overnutrition. It is generally known that we are in the midst of an epochal transition from an era of food scarcity and heavy muscular labor to one of an overabundance of food (except in the poorest classes), cheap inanimate power, and universal machine production. Moreover, variety, attractiveness, and ease of preparation of foods are increasing (11). Since feeding instincts developed under conditions of scarcity, the present abundance of food and lack of muscular exercise lead to

<sup>1</sup> Cf. Bancroft's (9) theory that sodium rhodanate (sodium thiocyanate?) when taken "about a teaspoonful a week of 10% solution," "will retard the aging of the colloids of the body."

overfeeding with tendencies to hyperglycemia, hyperlipemia, and obesity.

The islands of Langerhans may conceivably respond in two opposite ways to a persistent hyperglycemia. First, they may meet the hyperglycemia by hypertrophy with hyperinsulinism and establishment of a vicious circle: the more food, the greater the hyperinsulinism, and the greater the hyperinsulinism, the greater the craving for food. Obesity is of course the result. Ogilvie (10) compared the islands of Langerhans from 19 human cases of non-diabetic obese types with those from 19 cases of lean types. Sixty-eight per cent of the obese were found to possess (a) an abnormally high percentage of islet tissue area; (b) increased numbers of islands per given area, and (c) islands the average area of which was definitely greater than normal. Second, the islands may degenerate on account of hyperglycemia. Experiments by Hess (4) on overfeeding of fish (rainbow trout) are suggestive in this connection. Fish fed with beef liver, beef heart, or pig spleen had an average of 74 islands; fish fed with liver, heart, or spleen supplemented by 10 per cent lard or by lard and cooked cornstarch averaged 26 islands. This reduction was associated with fatty infiltration into the pancreas and lessened blood supply to this organ. Cataract was found only in fish with low islet counts. Elimination of lard and starch from the diet and increased exercise brought an increase in the islet count and disappearance of fat from the liver and pancreas. Hess suggested that islet reduction may be due to fatty infiltration and lessened blood supply to the pancreas, and that the same factors that cause islet reduction may also cause cataract.<sup>2</sup>

The following statistical reports tend to substantiate the idea that diabetes might be developed by overfeeding. Joslin, Dublin & Marks (13) reported that the rate of death from diabetes among women of age 45 and over doubled between 1920 and 1930. In men the increase was also considerable but not as great as in women. They also showed that two-thirds of the patients whose diabetes begins at 45 are at least 20 per cent above the average weight for their height and age, and nearly two-fifths of them are 20 per cent or more above

<sup>2</sup> The foregoing results do not indicate that diabetes in humans is established in a similar manner, or that established diabetes in humans can be cured by muscular exercise. In fact experiments by Soskin *et al.* (12), on men aged 20 and 32 years, showed that muscular exercise did not increase glucose tolerance or decrease insulin requirements.

normal weight. Joslin's (14) 1926 data illustrate the situation in patients prior to onset of diabetes.

Age Group	Total Number	Underweight	Overweight	Normal Weight
31-40 .....	172	8	153	11
41-50 .....	244	7	207	30
51-60 .....	252	2	220	30
61-70 .....	79	5	66	8
71-80 .....	14	1	11	2

Tyner (15) reported that 50 per cent of persons in the prediabetic state were obese, 25 per cent normal in weight, and 25 per cent underweight. Britten (16) reported that the adjusted mortality among diabetics per 100,000 of population in 1930 was 16.2 in the presumably well-fed "proprietors, managers and officials" as compared to 4.3 in agricultural workers and 10 to 13 in various classes of city workers (who presumably consume more food in comparison to their needs than agricultural workers). Hoffman (17) reported that the mortality from diabetes in fifty American cities increased from 15.9 per 100,000 in 1912 to 26.3 in 1932. A tabulation of sugar consumption per capita indicates that higher death rates coincide with excessive sugar consumption. It would perhaps be more proper to correlate the trend toward diabetes with the trend of the ratio of energy intake to energy expenditure.

*Is atherosclerosis a disease of overnutrition?*—The preceding section indicated that hyperglycemia (due to overfeeding on carbohydrates) tends to result in diabetes; this section indicates that hyperlipemia and especially hypercholesteremia (usually due to overfeeding on cholesterol-rich diets) tends to result in atherosclerosis.

The literature on arteriosclerosis was recently surveyed in a 700-page book by 24 distinguished pathologists under the editorship of Cowdry (3). Wise & Minot wrote the chapter on nutritional aspects of arteriosclerosis but almost every one of the other contributors had something to say on this problem. Anitschkow, who largely developed the experimental technic for producing atherosclerosis in rabbits, wrote the chapter on experimental production of atherosclerosis. H. Fox wrote on comparative mammalian and avian arteriosclerosis; P. Stocks on racial and climatic factors; W. Ophüls on pathogenesis especially as related to age; G. D. Williams on heredity; J. H. Wycoff on treatment; H. Gideon Wells on chemical changes. Aschoff and A. E. Cohn, respectively, introduced and summarized this valuable survey. These reviewers differ widely in their conclu-

sions concerning the influence of diet on the development of arteriosclerosis.

We shall review several papers which appeared after the publication of Cowdry's book. Of these the outstanding one is by Leary (2). According to Leary, atherosclerosis is responsible for 95 per cent of non-syphilitic lesions of visceral arteries, especially of the coronary, cerebral, and renal vessels. He believes that atherosclerosis is a nutritional disease amenable to prophylaxis and nutritional treatment, and that it is not an inevitable consequence of age since it appears in the young.

Leary compared atherosclerosis in the coronary arteries of rabbits produced by Anitschkow's method of feeding cholesterol with that found in humans. The fibrosis reaction in young rabbits corresponded to that found in young humans. There was substantial agreement between the experimental results of cholesterol feeding in rabbits and those observed in humans following high-fat diets. Fibrosis is therefore a reaction of youth and not of species. The evolution of lesions (lipoidosis, lipoid cell formation, fibrosis) is the same in rabbits as in humans, but lesions can be produced more rapidly in experimental animals. Studies on atherosclerosis, particularly of the coronary arteries, have shown that: (a) the process is primary in the intima; (b) the lesions are due to the presence of lipoids contained within macrophages which probably arise from the subendothelial layer of the intima; (c) the characteristic lesion in youth is a fibrosis associated with the presence of lipoid cells which do not accumulate in large aggregations because of the growth of fibrous tissue; the characteristic lesion with advance of age is an accumulation of large collections of lipoid cells with minimum connective tissue support and little or no physical support. As a result of poor nutrition, massive necrosis occurs. Necrosis of the cells frees the lipoids whose esters are split, and cholesterol, which is the dominant lipoid, crystallizes out locally. The cavity resulting from the necrosis, filled with cellular detritus, cholesterol crystals, fluid, and living lipoid cells, is the atheroma. In the coronary arteries, narrowing of the lumen (by fibrosis in the young or atheromatosis in the old) may reach a point where circulation is inadequate, particularly under conditions of stress, and death may result suddenly. The mush-like, semi-solid contents plug the narrowed channel and lead to thrombosis. (d) The usual cause of death in the young is thrombosis following subendothelial necrosis which extends to the endothelium. The usual cause

of death at older ages is the rupture of an atheromatous "abscess" into the lumen, usually followed by thrombosis. (e) The disease is not inflammatory in origin. There is no infection.

According to Leary: only humans die in early life from coronary sclerosis; only humans suffer almost universally from atherosclerosis with advance of age; only humans consume *regularly* during adult life cholesterol-rich foods (e.g., eggs); cholesterol furnishes a stable frame work for rapidly multiplying cells (as in the chick embryo) but its consumption during later years becomes increasingly menacing, because cholesterol is not combustible, its avenues of excretion are limited, and it tends to be stored. As in diabetes, the inheritance of a poor cholesterol metabolism appears to be associated with the tendency to an early death from coronary sclerosis. When very high fat diets were advocated for diabetics, there was such an increase in arteriosclerosis that the question arose whether this was due to the diabetes or to its treatment. Xanthomas were common, an X-ray examination of the legs of diabetic children demonstrated in some cases calcification of the arteries, the late stage of arteriosclerosis. Under modern diets containing less fat, the excess of arteriosclerosis in diabetics has declined, xanthomas are no longer found, and X-ray examination of the legs of diabetic children is negative.

Another interesting paper is by Rosenthal (18). This author, like Meeker & Jobling (19) and Leary, accepts Aschoff's infiltration theory, that cholesterol esters along with other lipoids are deposited in the aorta from the blood without selection. Later the cholesterol esters are split, leaving increasing amounts of free cholesterol. The cholesterol and total lipid deposits are proportional to the severity of lesions so that age is not the only factor involved.

Rosenthal quotes from Raab's interesting observations showing the incidence of atherosclerosis and blood pressure in various peoples and presenting estimates of proteins, fats, and carbohydrates in their diets. From this table he concludes that: (a) in no people for whom a high cholesterol (in the form of eggs, butter, and milk) and fat intake are recorded is atherosclerosis absent; (b) where a high protein diet containing little cholesterol and little fat is consumed atherosclerosis is not prevalent; (c) neutral fat in the diet paves the way for cholesterol absorption. Diets high in cholesterol and low in neutral fat may result in lower blood cholesterol than diets high in neutral fat and low in cholesterol. In this connection, Williams, Anderson & Mendel (20) found that increase of cholesterol in livers (and less

definitely in plasma) of rats fed 1 per cent cholesterol ran parallel with the total dietary lipoids. "In the high fat diets the addition of cholesterol caused an increase of 20 to 30 per cent in weight of liver." Aschoff traced the marked decrease of atherosclerosis in Central Europe following the war to low fat intake, just as the decrease of diabetes during the war was traced to low carbohydrate intake. Ismail (21) reported that his well-to-do private patients in Egypt subsisting on European diets showed a high incidence of atherosclerosis, while his hospital patients, subsisting largely on carbohydrates, showed a low incidence of atherosclerosis. Donnison found that Negroes in East Africa (subsisting on cholesterol-poor diets) had a negligible incidence of atherosclerosis; Rosenthal found that Negroes in Chicago (subsisting on cholesterol-rich diets) had a high incidence. This, according to Rosenthal, indicates the possibility that cholesterol metabolism plays a rôle in the development of atherosclerosis. The influence of hypercholesteremia appears to be cumulative. Thus feeding massive doses of cholesterol to rabbits, with accompanying hypercholesteremia, results in a rapid production of atherosclerosis; on the other hand, Anitschkow produced a slow-developing atherosclerosis in rabbits without hypercholesteremia by feeding eggs and milk (over a period of  $2\frac{1}{2}$  years). High blood pressure may favor development of atherosclerosis by forcing the lipoids into the aorta at a greater rate, or by hastening the aging process, stretching the vessels, and decreasing their elasticity. Aging of the colloids and decreasing elasticity of vessels favor binding or precipitation of lipoids, and decrease their power of forcing out the lipoids so that deposition of cholesterol becomes irreversible. Anitschkow and others hastened the experimental development of atherosclerosis in cholesterol-fed rabbits by adding to the diet blood-pressure elevating substances, as epinephrin; but increasing the blood pressure without feeding cholesterol did not lead to atherosclerosis. While increasing age and blood pressure favor development of atherosclerosis, atherosclerosis is relatively common at early ages with normal blood pressures. Aging in itself does not lead to atherosclerosis but to a pure senile sclerosis. If the reviewer understands it correctly, deposition of lipoids is autocatalytic: the more deposition, the greater the irritation set up by the deposit, and the greater the blocking of the blood supply to the part. These in turn favor more deposition of lipoids as also of hyalin and calcium. Cholesterol-rich diets, high blood pressure, and advancing age accelerate this process.

An experiment of a different type is of interest in this connection. McCay *et al.* (22) devised a synthetic diet (regenerated cellulose, 20; cornstarch, 40; casein, 15; sucrose, 10; yeast, 5; lard, 7; salts, 4; cod-liver oil, 2; orange or tomato juice) for herbivores. Rabbits, guinea pigs, and especially goats fed this diet, developed pathological changes in the skeletal and heart muscles detected on gross or histological examination. In many cases the goats grew well and appeared well for 2 to 3 months, but, when excited, they dropped dead. The authors partly attributed these degenerative changes to the cod-liver oil of the diet, probably to the fatty acids rather than to the fat-soluble vitamins. The rabbits lived longer on this diet containing no fat-soluble vitamins than upon one including 2 to 3 per cent cod-liver oil (i.e., within the limits of cod-liver oil commonly fed to children). As cod-liver oil is rich in cholesterol, it would have been instructive to examine the circulatory system for evidences of atherosclerosis.

Other papers that might be discussed if space permitted are: Raab (23) who concluded that long-continued feeding of foods rich in cholesterol, vitamin D, and fats (eggs, cod-liver oil, and other animal fats) may produce arteriosclerosis in man; Agduhr & Anderson (24) who produced lesions in the heart and elsewhere in white mice by feeding 30 cc. of cod-liver oil per kilo of body weight; Hunt (25) who found a decline in blood cholesterol on omitting eggs from the diet of diabetic children; White & Hunt (26) who found that over-nutrition is accompanied or followed by an increase of cholesterol in blood; Rabinowitch (27) who concluded that the cardiovascular and renal complications in diabetes are due to the high-fat diet, and who, on instituting a radical reduction in fat (50 to 100 gm.), a corresponding increase in carbohydrates (up to 300 gm.), and low caloric intake generally, obtained favorable results (in his experiments in which carbohydrate was increased from 89 to 246 gm. and energy intake reduced from 1906 to 1774 Cal. the plasma cholesterol was reduced from 0.257 to 0.187 per cent); Twiss & Greene (28) who discussed cholesterol metabolism in hepatic disease, disturbance of cholesterol metabolism in obesity (hypercholesteremia is produced not only by cholesterol-rich foods but also by any fat-rich foods); Page & Menschick (29) who compared the cholesterol content in organs of rabbits fed on customary diets and those fed cholesterol in addition; Turner (30) who found interesting results on the influence of administration of potassium iodide on development of atherosclerotic lesions in cholesterol-fed rabbits; Ham & Lewis (31) who produced widespread



and severe intimal lesions of the coronary arteries in young rats by administrations of heavy doses of irradiated ergosterol; Mosebach (32) who found that experimental arteriosclerosis in rats is produced only by a combination of administrations of cholesterol, excessive functional activity, and injury to the vascular wall; Okey *et al.* (33) who found that dietary administration of 1 per cent cholesterol to rats caused a large increase in blood lipoids, particularly cholesterol and cholesterol esters; Kanócz & László (34) who claim that the customary rise in blood pressure is prevented if lecithin is simultaneously administered with the cholesterol, in the experimental production of atherosclerosis in rabbits; Chanutin & Ludewig (35) who produced fatty livers by feeding a diet containing 2.5 per cent cholesterol; Blatherwick *et al.* (36) who produced cholesterol-rich fatty livers in rats by feeding liver, or by adding 1 per cent cholesterol to a mixed diet (supplementing the diet with 2 per cent lecithin did not prevent this development); Best *et al.* (37) who found that a diet containing 40 per cent beef fat or 1 per cent cholesterol results in formation of fatty livers, but that administration of 1 per cent choline, or betaine, tends to prevent this formation (choline occurs in tissues generally as lecithin and sphingomyelin); Kirchgessner (38) who reported that ingestion of 85 gm. of cholesterol by rabbits over a period of 2 months caused atheromatous changes in the vessels, especially in the aorta, resembling those of human atherosclerosis, and accompanied by a 23-fold increase in blood cholesterol; Sperry & Stoyanoff (38a) who found that cholesterol-fed rats grew less well, ate less food, grew less efficiently (on account of deposition of cholesterol esters in all tissues but especially in liver) than control rats not receiving cholesterol supplements, and that chickens behave like mammals in absorbing cholesterol and depositing it in the liver, but they differ from rats in depositing proportionately more free cholesterol.

The foregoing discussion indicates that chronic overfeeding of lipoids and carbohydrates tends to shorten the life span. With the exception of vitamin D there is probably no danger from overfeeding vitamins. On the contrary the well-known researches from Sherman's (39) laboratory point to favorable results from liberal supplies, particularly of vitamin A. Thus rats fed for 8 generations on diet B consisting of one-third whole milk powder and two-thirds whole wheat not only reproduced and grew better, but also lived longer than rats fed on diet A consisting of one-sixth whole milk powder and five-sixths whole wheat. The following table compiled from Sherman's

papers contradicts somewhat McCay's conclusions that rapidity of growth and longevity are incompatible.

	Weight in Grams						Average Duration of Life, Days	Percentage of Survival, Days									
	At 28 Days		Gain, 28 Days after Weaning														
								600		700		800		900		1,000	
	M	F	M	F	M	F		M	F	M	F	M	F	M	F	M	F
Diet A, 1/6 milk....	34.3	33.8	43.2	37.9	571	608	42.9	54.1	14.8	27.6	2.9	12.2	0.0	2.6	0	0.5	
Diet B, 1/3 milk....	41.8	40.1	51.1	52.3	635	669	65.3	73.0	32.3	43.6	10.5	15.9	1.6	5.5	0	7.2	

However, in their latest communication Sherman & Campbell (39) say: "Systems of feeding which influence the rate of growth may influence the length of life in either direction"; "Among individuals of the same heredity and sex, living in the same environment and eating *ad libitum* of the same food, rate of growth and length of life vary independently of each other."

#### FEEDING STANDARDS

Brody, Procter & Ashworth (40) reported that the basal energy metabolism of mature animals ranging in weight from 0.02 to 4000 kg. (mice to elephants) increases with the 0.73 power of body weight ( $Q = 70.5 M^{0.734}$ , in which  $Q$  is heat production in kilo-calories per day and  $M$  is body weight in kilograms). The endogenous urinary nitrogen excretion by mature animals ranging in weight from 0.02 to 500 kg. (mice to cattle) tends to increase with the 0.72 power of body weight ( $N = 146 M^{0.72}$ , in which  $N$  is the endogenous urinary nitrogen excretion in milligrams per day and  $M$  is the body weight in kilograms). The difference between the above exponents, 0.734 and 0.720, is well within the limits of experimental error, so it may be concluded that both basal metabolism and endogenous nitrogen follow the same course with increasing body weight. On the basis of these facts they suggested tentative maintenance standards for all warm-blooded animals. The feeding standards were computed from the equation  $Y = AM^{0.73}$ , in which  $Y$  is digestible food requirement for maintenance of body weight  $M$ . For digestible energy (calories), the value of  $A$  is double the basal metabolism; for digestible protein, the value of  $A$  is four times the protein equivalent ( $N \times 6.25$ ) of the endogenous urinary nitrogen. The computed values agree with current feeding practices. The new features about these standards are: the ratio of protein to energy is the same for the maintenance of all species

(approximately 10 per cent of the calories are in the form of protein, or the "nutritive ratio" is about 1 : 8.7) ; the protein and energy allowances increase not directly with body weight, as is the present custom, but with the 0.73 power of body weight. That is to say, increasing body weight by 100 per cent increases the food needs not by 100 per cent but only by about 70 per cent. In the reviewer's opinion the value of *A* (absolute maintenance level) is likely to change considerably, but the relative needs of animals of different body weights—the exponent, 0.73, in the equation—are not likely to change appreciably.

Stiebling & Ward (41) representing the Bureau of Home Economics, United States Department of Agriculture, and apparently this department's Program Planning Division of the Agricultural Adjustment Administration, prepared a series of nutrition standards for humans which promise to have considerable historical interest. Their paper presents four dietary plans: (a) "restricted diet for emergency use," which allows a narrow margin of safety, costs 17 cents per capita per day, and requires 1.2 acres (exclusive of grazing land) per capita per year to produce; (b) "adequate diet at minimum cost," which costs 24 cents per day and requires 1.5 acres per capita per year; (c) "adequate diet at moderate cost," which costs 42 cents and requires 1.8 acres per capita; and (d) "liberal diet," which costs 51 cents and requires about 2.1 acres. The cost of diets refers to average retail prices in the United States during 1931-32. In terms of cost per average family (2 moderately active adults, three children aged 2, 5, and 13 years) the cost varies from \$22.85 to \$59.48 per month; or on a yearly basis the cost of diet (a) is estimated at \$350, diet (b) \$500, diet (c) \$800, diet (d) \$950. It is assumed that the food would be prepared at home, no allowance being made for meals at restaurants. The following is a concrete illustration of a week's supply of foods required as a "restricted diet" for a family of five (two adults, three children under 13 years): 14 quarts of milk, 9 eggs, 2½ lbs. of meat, 4 lbs. of butter and other fats, 2¼ lbs. of dried beans and peas, 3 qts. of tomatoes, 3 lbs. of cabbage and similar green or yellow vegetables, 1 lb. of dry or 5 lbs. of fresh canned fruit, 3 lbs. of other fruits or vegetables, 16 lbs. of potatoes, 24 lbs. of flour and cereals, and 4½ lbs. of sugar and other sweets.

The essential difference between the four nutritive levels consists in varying the ratio of grain products, dried beans, and potatoes to animal products, vegetables, and fruits, as illustrated by the following condensed table:

Foodstuffs	Percentage Distribution of Foods on the Four Diets							
	Restricted Diet for Emergency Use		Adequate Diet at Minimum Cost		Adequate Diet at Moderate Cost		Liberal Diet	
	Monetary Distribution	Calorie Distribution	Monetary Distribution	Calorie Distribution	Monetary Distribution	Calorie Distribution	Monetary Distribution	Calorie Distribution
Grain products, dried beans, and potatoes .....	20	43	15	35	10	24	5-7	15
Dairy products .....	25-30	12	30-35	18	25-30	19	30	19
Vegetables and fruits .....	20-25	14	20-25	15	25-30	18	30	18
Lean meat, fish, and eggs.....	10	5	15	8	15-20	12	25-30	21
Fats, sugars, accessories.....	20	26	15	24	15-20	27	5-7	27

The following condensed table of average absolute amounts of foodstuffs allowed per average person per year for the 4 dietary levels will bring out the relations in a more useful manner:

Foods or Nutrients	Restricted Diet for Emergency Use	Adequate Diet at Minimum Cost	Adequate Diet at Moderate Cost	Liberal Diet
<b>Per year</b>				
Grain products,* lbs. ....	240	224	160	100
Dried beans, peas, nuts, lbs. ....	30	30	20	7
Potatoes and sweet potatoes, lbs. ....	165	165	165	155
Milk, quarts (or equivalent).....	155	260	305	305
Lean meat, poultry, fish, lbs. ....	30	60	100	165
Eggs, dozen .....	8	15	15	30
Tomatoes and citrus fruits, lbs. ....	50	50	90	110
Leafy green and yellow vegetables, lbs. ..	40	80	100	135
Dried fruits, lbs. ....	10	20	25	20
Other vegetables and fruits, lbs. ....	40	85	210	325
Fats (oils, butter, bacon, salt pork), lbs.	45	49	52	52
Sugars, lbs. ....	50	35	60	60
<b>Per day</b>				
Calories .....	2,675	2,980	2,965	2,900
Fat, gm. ....	87	115	139	149
Carbohydrates, gm. ....	398	397	370	310
Calcium, gm. ....	0.85	1.28	1.26	1.27
Phosphorus, gm. ....	1.84	1.72	1.58	1.61
Iron, gm. ....	0.0111	0.0134	0.0144	0.0152
Vitamin A units.....	2,746	5,067	5,692	6,495
Vitamin C units.....	86	118	168	206
Calories from protein, percentage.....	11	12	11	12
Protein from animal products, percentage .....	25	29	47	66

\* The annual per capita grain consumption between 1925 and 1929 was 220 pounds gross (including a 20 per cent waste) or 187 pounds net. In this table only 5 per cent waste is allowed.

The diets were formulated in accordance with the following "standards":

	Dietary "Standards" per Capita per Day						
	Energy	Protein	Calcium	Phosphorus	Iron	Vitamin A Units	Vitamin C Units
	Cal.	Gms.	Gms.	Gms.	Gms.		
Child under 4 years.....	1,200	45	1.00	1.00	.006-.009	3,000	75
Boy, 4-6; girl, 4-7 years.....	1,500	55	1.00	1.00	.008-.011	3,000	80
Boy, 7-8; girl, 8-10 years.....	2,100	65	1.00	1.00	.011-.015	3,500	85
Boy, 9-10; girl, 11-13 years.....	2,400	75	1.00	1.20	.012-.015	3,500	90
Moderately active woman; boy, 11-12; girl, over 13 years.....	2,500	75	1.00	1.20	.013-.015	4,000	95
Very active woman; active boy, 13-15 years.....	3,000	75	0.88	1.32	0.015	4,000	100
Active boy over 15 years.....	3,000-4,000	75	0.88	1.32	0.015	4,000	100
Moderately active man.....	3,000	67	0.68	1.32	0.015	4,000	100
Very active man.....	4,500	67	0.68	1.32	0.015	4,000	100

Dietary levels (c) and (d) furnish nutrients in excess of the "standards," especially vitamins and minerals; diet (b) and especially (a) fall below the standard, particularly in vitamins, iron, and calcium.

It is instructive to quote in this connection the revised (1934) nutrition standards prepared by the British Ministry of Health (42). The revision was made by the Ministry's Advisory Committee on Nutrition and representatives of a committee appointed by the British Medical Association.

Individuals	Calories, per day
Child 1-2 years.....	900-1,100
Child 2-3 years.....	1,100-1,400
Child 3-6 years.....	1,400-1,700
Child 6-8 years.....	1,700-2,000
Child 8-10 years.....	2,000-2,300
Child 10-12 years.....	2,300-2,800
Child 12-14 years.....	2,800-3,000
Girl 14-18 years.....	2,800-3,000
Boy 14-18 years.....	3,000-3,400
Woman, housewife.....	2,600-2,800
Woman, active work.....	2,800-3,000
Man, light work.....	2,600-3,000
Man, moderate work.....	3,000-4,000
Man, heavy work.....	3,400-4,000

The energy requirement per man per day for the entire population is put at 3,000 Calories.\* The protein requirement is considered to be 80 to 100 gm. per day, of which not less than one-third must be of animal origin. Emphasis is placed on the importance of milk as a food for the child and the nursing and expectant mother, because it is the "only naturally balanced food containing as it does not only first-class protein (18.7 gm. or two-thirds ounce to the pint) but also minerals, vitamins, carbohydrate, and fat."

\* One Calorie is equal to one kilogram-calorie, or 1,000 calories.

The following are abstracts of the discussion given by Stiebling & Ward. The grain products, especially if unmilled and supplemented by milk, are inexpensive sources of energy, protein, phosphorus, and iron. Milk is an inexpensive source of calcium, phosphorus, high quality protein, and vitamins A and G, supplying cheaply what the grains lack; hence the dietetic importance of the cereal-milk combination in the low-cost but adequate diets. It is advised that children should have one quart of milk per day. Dried legumes are important sources of energy, calcium, iron, vitamin B, and protein, which is of good quality in soybeans but not so good in other legumes. Green leafy vegetables (spinach, kale, collards, turnip greens, beet tops, mustard greens), peas, asparagus, and snap beans, are economical sources of iron, and vitamins A and G. Carrots, apricots, and other colored (orange or yellow) fruits or vegetables furnish vitamin A. Tomatoes, raw cabbage, and citrus fruits are rich sources of vitamin C. Cod-liver oil (2 to 4 teaspoonsful per day) is included for young children (under 2 years) during the winter months. Most adults in temperate zones get enough sunshine so that the needed amounts of vitamin D are provided by the activation of ergosterol in the skin by ultra-violet rays. Lean meats, eggs, and liver are recommended for their flavor and iron, liver also for vitamins A and D. The daily requirement for vitamin C is probably covered by the quantity contained in an ounce of orange, grape fruit, canned tomato, or raw cabbage (15 Sherman units). However, a more liberal intake is advantageous. Five hundred to 1000 Sherman-Bourquin units of vitamin G per person per day is furnished by milk, green leafy vegetables, eggs, and lean meat.

It is estimated that if milk consumption per capita were to approach the minimum consumption of 260 quarts per year as recommended by Stiebling & Ward then instead of there being surplus cattle, there would be a shortage of 15 million dairy cows (43). Likewise there would be underproduction of vegetables, fruits, meat, poultry, and eggs (44). Farm activities would have to be increased 70 to 80 per cent in order to supply the "liberal diet" for the entire population. This statement indicating the existence of undernutrition with respect to green vegetables, fruits, and dairy products, especially among the under-privileged children, does not contradict a statement in the preceding section pertaining to the existence of overnutrition with respect to carbohydrates and fats, especially among the middle and upper class adults. Besides, increasing con-

sumption of vegetables and fruits tends to decrease the total energy intake.

#### PROBLEMS IN ENERGETIC EFFICIENCY

*Definitions.*—In last year's *Review* (45) gross (or total) efficiency<sup>a</sup> was conventionally defined by the ratio,

$$\frac{\text{energy in product}}{\text{available energy in food}},$$

and net (or partial) efficiency by the ratio,

$$\frac{\text{energy in product}}{\text{available energy in food less energy of maintenance}}.$$

Palmer & Kennedy, and Morris, Palmer & Kennedy (47), in their interesting studies on variations and inheritance of growth efficiency, used an "efficiency quotient" which may be briefly defined by the ratio,

$$\frac{\text{food consumed}}{\text{gain in weight} \times \text{weight} \times 100}.$$

This is a confusing index of efficiency because it represents (unwarrantedly) efficiency as an inverse function of weight and it is a reciprocal of efficiency as defined by chemists, complicated however by multiplying the gain in weight by weight. Kleiber (48) analyzed the Palmer-Kennedy efficiency quotient as follows: he computed the gross and net growth efficiencies of 65-gm. and 6500-gm. animals; the gross efficiency was the same in both animals (34 per cent), and the net efficiency was the same in both animals (70 per cent). But he found the efficiency quotient of Palmer & Kennedy to be 4.5 for the 65-gm. animal and 0.045 for the 6500-gm. animal! Kleiber also worked over the data on growth of rats given in table 3 of the paper by Morris, Palmer & Kennedy. In the case of males the efficiency quotient decreased with increasing age, leading to the improbable conclusion that the conventional efficiency of growth, weight gained to food consumed, increased with increasing age; in the case of the females, the efficiency quotient declined from the first to the second week, then rose slightly. Kleiber's analyses show that the gross effi-

<sup>a</sup> See Needham (46) for a historical discussion of efficiency definitions.



ciency declined uniformly with age in both males and females and the net efficiency remained constant for the males, but declined somewhat in the females with increasing age (due apparently to greater deposition of fat and lowered water content in the females than in the males). It would be instructive to recompute the data by Palmer *et al.* in terms of gross and net efficiency and in terms of Kleiber's "relative food level,"  $U/B$ , defined by the ratio, food consumed to food expended for maintenance (49). The reviewer does not believe that there are individual or sex differences in net efficiency of growth, but there may be real differences in gross efficiency due to differences in food consumption above maintenance, or apparent differences due to differences in composition of the gains. It need hardly be said that the greater the amount of food consumed above maintenance the greater will be the food utilized for productive purposes in comparison to mere maintenance, and hence the greater the gross (or total) efficiency of the animal.

*Influence of body size on efficiency.*—The difficulty in evaluating the influence of size on gross efficiency is that conditions not directly related to size often influence efficiency to a greater extent than does size. Thus, as pointed out by Kleiber (48), the milk-producing efficiency of a dairy cow is greater than of a beef cow, but this is not a size effect. The growth efficiency of humans is less than of farm animals not because humans are larger or smaller but because they grow more slowly, thereby expending more energy for the overhead cost of maintenance. The egg-producing efficiency of a large hen may be less than of a small hen of the same breed not because she is larger but because she is fatter. Kleiber (49) concluded on the basis of theoretical considerations that the energetic efficiencies of animals are the same if their  $U/B$  values (ratios of "available" food consumed,  $U$ , to basal metabolism,  $B$ ) are the same.<sup>4</sup> He then presented data showing that the difference between the  $U/B$  ratios of two steers is greater than between steers, chicks, and rabbits ( $U/B = 4.43$  for a group of chicks; 5.03 for a rabbit; 4.24 and 5.57 for two steers). Hence he concluded that the growth efficiency of steers is (within the limits of variability of animals within a species) the same as of chicks, rabbits, and very likely of all warm-blooded animals, provided that factors other than weight are the same.

<sup>4</sup>  $U$ , "available" food, is the metabolizable energy of the food, that is gross energy of the food less the energy of the feces and urine.

Data accumulated in the reviewer's laboratory since writing last year's review (45) substantiate the theory that gross energetic efficiency tends to be independent of live weight. Thus (50), 600-pound ponies as well as 1500-pound draft horses approached the same maximum gross efficiency, namely 25 per cent (or net efficiency of 34 per cent). Data on the efficiency of milk secretion (51) were not quite as constant. Data were analyzed on 243 dairy cows of 5 years or over. After appropriate corrections for changes in body weight during lactation periods of about one year, the *gross* efficiency (ratio of energy in milk to energy of digestible nutrients consumed, assuming 1 gm. digestible nutrients is equivalent to 4 Cal.) declined from 32.5 per cent at body weight 700 pounds to 30 per cent at body weight 1000 pounds; it remained practically constant between body weights 1000 to 1500 pounds. The *net* efficiency remained constant at about 61 per cent. The difference in gross efficiency between the large and small cows was comparatively small at best, and may well have been due to genetic and management factors rather than to size as such. These results must be considered as tending to substantiate rather than to contradict Kleiber's conclusions of the independence of efficiency from body size.

Incidentally, the net efficiency of milk production (60 to 70 per cent) was the same as that reported by Needham (46) and by Kleiber (48, 49) for growth, and is about twice as great as for work at maximum rate (pulling loads on a horizontal plane) by horses (50). The gross efficiency must increase with increasing level of milk flow, but it happened to be of the same order as the gross efficiency of growth of baby chicks (34 per cent) as reported by Kleiber (49) and for other species as reported by Needham (46).

*Paired versus ad-libitum feeding methods.*—The paired-feeding method (52) consists in "pairing" animals *A* and *B* to the same amounts of food energy, varying the ration only with respect to another nutrient, *X*, under investigation. The relative rates of growth of *A* and *B* are commonly taken as criteria of the deficiency (or excess) of *X* on nutritive responses. From the standpoint of energetic growth efficiency one wonders what becomes of the energy consumed by *B*, which in the case of *A* was used for growth. Some reports indicate that differences in weight increments on equicaloric diets are not due to differences in gains of energy but of water, and, of course, of minerals and nitrogenous substances accompanying the extra water. Thus Lee & Schaffer (53) reported a study on the com-

position of gains of pair-fed rats, the growth of one member of the pair was greatly accelerated by injection of anterior pituitary growth hormone. The rapidly growing animals were found to contain more water and protein and less fat and energy than the smaller controls. Hogan, Johnson & Ashworth (54) paired rats to equicaloric diets differing in protein content. The high-protein diet contained 24 per cent casein and 46 per cent starch while the low-protein diet contained 6 per cent casein and 64 per cent starch. Each mixture was supplemented by lard, 12.5; cod-liver oil, 2.5; yeast, 8.0; mineral mixture, 4.0; bone ash, 3.0. The low-protein and high-protein rats expended the same amounts of energy for maintenance as determined by keeping them in Haldane chambers. There was no difference in energy stored, but the rats on the high-protein diet gained more weight (43.9 gm. as compared to 27.6 gm.), more water (30.1 gm. as against 16.4 gm.), more protein (9.1 gm. as against 4.7 gm.), more ash (1.7 gm. as against 1.0 gm.), but less fat (2.8 gm. as against 5.2 gm.) than the low-protein rats. In brief, the low-protein rats were smaller but fatter and so stored as much energy as the larger but more watery high-protein rats. Braman, Black, Kahlenberg, Voris, Swift & Forbes (55) found that rats fed on yellow corn gained more weight than rats fed on equicaloric white corn but both stored the same amount of energy; the heavier yellow-corn rats simply contained more water. Forbes *et al.* (55) paired rats to equicaloric diets differing in vitamin-A content. The difference in weight gain, in favor of the higher vitamin-A diet, was statistically significant, but the difference in energy storage was not; the more rapidly growing animals simply had a higher water and nitrogen content.

Other reports indicate differences in energy storage on equicaloric diets. Swift, Kahlenberg, Voris & Forbes (56) paired rats to equicaloric diets containing (a) 8 per cent milk proteins and (b) 8 per cent milk proteins supplemented by 0.24 per cent *l*-cystine, with the following results: The rats on the cystine-supplemented diets made greater gains in weight, and stored 40.4 more Calories (10.8 per cent more energy) and 24.4 per cent more nitrogen than did the controls. The extra storage of 40.4 Cal. by rats receiving the cystine supplement was accounted for by diminished losses of 2.8 Cal. in urine, 8.6 Cal. in feces, and 29 Cal. in heat production. Statistical analyses indicated that these absolute differences in losses were highly significant. It is instructive to express the same losses in terms of percentages of food energy consumed, in which case they are: 0.1 per cent

in urine, 0.3 per cent in feces, and 1.0 per cent in heat production (maintenance, activity, specific dynamic action). In other words the difference in utilization (not counting energy cost of maintenance) is 0.4 per cent of the food. This difference, while significant statistically, may not be so physiologically, especially in view of the fact that the pace-maker of the pair having the lesser appetite is likely to waste more of its food (perhaps up to 0.4 per cent) in spite of all precautions. The difference of 1 per cent in heat production may likewise be insignificant, in comparison to possible overall experimental errors. McClure, Voris & Forbes (57) investigated the influence of moderate vitamin-B deficiencies on relative energy and protein utilization of rats paired to equicaloric diets. There was no statistical difference in the gains in weight of the paired rats, but there was a difference of 19.6 Cal. in energy stored in favor of the rat on the supplemented diet. This difference was accounted for by 8.8 Cal. greater urinary loss, 0.3 Cal. greater fecal loss, and 10.5 Cal. greater heat loss (maintenance, activity, specific dynamic action) by the rats on the deficient diet. In their research on moderate vitamin-D deficiency, Forbes *et al.* (55) found no difference between the paired rats as regards gains in body weight, but they found significantly greater storage of energy by the vitamin-D supplemented rats. This energy difference was accounted for by the greater heat production of rats on the vitamin-D deficient diet. Moderate vitamin-G deficiency reduced (in comparison to paired control) growth in weight, and in storage of protein and energy (55).

More striking, indeed spectacular, differences in growth and energy storage obtained by the paired-feeding method are indicated in data by Forbes (58) in a comparison of rations containing 25, 20, 15, and 10 per cent protein. Rats on the 25 per cent and 20 per cent protein diets gained 119 gm. in 12 weeks; on the 15 per cent protein diet, 107 gm.; on the 10 per cent diet, 79 gm. Energy stored on the 25, 20, 15, and 10 per cent protein diets was respectively 302, 296, 263, and 217 Cal. Heat production on the 25, 20, 15, and 10 per cent protein diets was respectively 1626, 1642, 1668, and 1709 Cal.<sup>5</sup> Nitrogen stored was respectively 4.17, 4.08, 3.68 and 2.51 gm. Digestibilities of energy were respectively 93.4, 92.9, 91.8, and 91.5 per cent; of nitrogen, 94.2, 93.3, 91.8, and 88.1 per cent. But even

<sup>5</sup> These results as regards energy storage and heat production contradict aforecited results reported at the same meeting by Hogan, Johnson & Ashworth (54).

in the case of these large differences it is not easy to interpret the results. To cite typical difficulties: on a *a priori* consideration one would assume that animals having higher protein percentages would have higher heat production since: (a) they gained more weight (and nitrogen), and therefore their maintenance metabolism should be higher; (b) they consumed more protein, and therefore their heat production due to specific dynamic effect should be higher; (c) their food intake was restricted to that consumed by the animals on the lower protein intake, and hence they would be hungrier, therefore more restless (that is, they would be on a higher activity level), and their energy expenses for activity would be expected to be higher. Actually the reverse was found to be the case; the heat production declined with increase of protein in the diet.

The method of measuring heat production in these excellent researches by Forbes *et al.* consisted in deducting the energy stored and excreted from the energy in the feed, as illustrated by the following example for a typical rat:

Energy of feed .....	2,275.6	Cal.
Energy of feces.....	171.6	Cal.
Energy of urine.....	75.6*	Cal.
Energy of fat stored.....	101.0	Cal.
Body gain in protein.....	88.9	Cal.
<hr/>		
Heat production (by difference).....	1,838.6	Cal.

\* 2.1 Cal. were added to the energy of the urine as representing the energy loss during drying (gm. N. loss  $\times$  5.447). All values with the exception of heat production were determined directly by a bomb calorimeter.

Hogan, Johnson & Ashworth (54), whose results contradict Forbes' results, computed heat production from carbon dioxide and oxygen data obtained by keeping the rats in Haldane chambers for 23¾ out of 24 hours.

The difficulty of interpreting differences in weight gains, whether the gains represent energy or water, suggests that the composition of these gains should be analyzed (59). But this cannot often be done when the experimental subjects are humans or even large farm animals. There are similar uncertainties concerning the composition of gains when using the *ad-libitum* feeding method, but differences in gains by the *ad-libitum* method are usually much greater and so more easily interpreted. When the paired-feeding method gives but slight differences in weight or in energy storage, as in the aforesaid corn and vitamin-A experiments, one would logically decide that

there are no significant differences in the nutritive values of feeds under comparison, a conclusion which is obviously incorrect. Use of proper criteria would demonstrate unmistakably the known superiority of yellow corn to white and the known necessity for vitamin A in growth.

Morrison (60) presented the following practical objections to the paired-feeding method in animal-production investigations:

The equalizing of feed intakes may defeat the very object of the experiment. If one ration is nutritionally deficient, even to a very limited extent, the first effect of such a deficiency is quite commonly to cause the animals fed this ration to consume less feed than they would on a nutritionally complete ration. If we then permit the animals on a good ration to eat no more feed than the animals on the poor ration, we penalize the efficient ration, and fail to take advantage of most of its superiority. If feed intake is equalized in such a case, a difference between the good ration and the poor ration will be shown only if there is a difference in utilization of food nutrients *at the restricted level of feed intake*. This is, however, probably not the chief difference between efficient and poor rations. In most cases the good ration is superior from the economic standpoint because it causes greater food consumption and greater production. This results in an increase in the percentage of nutrients used for production, due to the fact that a smaller proportion is needed for maintenance.

Mitchell (61) admits "the quite general failure of animals to consume unbalanced rations as avidly as balanced rations."

The aforementioned objections against the paired-feeding method do not oppose the broad generalization of Mitchell (61) and others that the degree of utilization of nutrient A depends on the simultaneous presence of all other nutrients, B, C, . . . required for a given result; since it is known that the amounts of B, C, . . . in a ration may be varied within limits without appreciably affecting the desired result. Many of the aforementioned paired-feeding experiments substantiate this conclusion.

The reviewer interprets the situation as follows: (a) The *net* (or partial) energetic growth efficiency tends to be constant, that is, independent of small variations in the dietary make-up. (b) The *gross* (or total) energetic growth efficiency depends on available energy consumed above maintenance. (c) A deficiency of an essential nutrient tends to reduce food intake, and thereby tends to reduce gross efficiency. (d) Animals paired to two equicaloric diets, one of which is deficient, are not likely to differ in energetic growth efficiency unless the deficiency decreases availability of energy at the given level of energy intake. In consequence the paired-feeding method as

currently used in the energetic sense is, in its simplest terms, a study of the influence of a dietary deficiency on availability of energy only at a given level of energy intake. (e) Differences in weight gains on equicaloric diets are not indicative of energy gains because they may be due to differences in composition of gains. (f) The principle of the paired-feeding method is sound; the soundness of its application depends on choice of a relevant criterion for measuring the attainment of the desired aim. If the aim is to evaluate the relative nutritionally available energy of equicaloric amounts of lard and crisco, or of lactose and sucrose, relative energy storage should be the criterion. If it is desired to evaluate the relative nutritional availability of calcium in raw and pasteurized milk (61a), the criterion should not be difference in energy storage (which would be irrelevant) but difference in calcium storage. If it is desired to evaluate the relative vitamin-B contents in rations the criterion should be relative food intake since differences in vitamin-B intake under certain critical conditions are followed by characteristic differences in food intake (61b). But, of course, using food intake as criterion is using the *ad-libitum* rather than the paired-feeding method. This example indicates that there is no sharp dividing line between these two methods and that there are occasions for using either one or both, depending on the aim in view.

The following notes are from Campbell (62). Results of the *ad-libitum* feeding method are interpreted by "the application of common sense" and use of statistical methods (standard deviation, probable error and coefficient of variation of the mean). Growth criteria for rats include rate of growth of the young during the first 28 days after weaning, gain in weight per 1000 Cal. of food consumed, gain per gram of protein consumed, calories of food consumed per gram of body weight per day, and average weights on the different diets at all ages. The reproduction criteria include ability to bear and rear young, average weight of young at weaning time, length of breeding period, and age at sexual maturity. A number of recent papers discuss methods of statistical analysis of data obtained by the *ad-libitum* method (63).

#### NITROGEN FACTORS IN NUTRITION

This section discusses one or two topics of interest in connection with last year's review (45) on energy factors in nutrition.



*The Terroine-Sorg-Matter Ratio.*—Terroine & Sorg-Matter (64) found that the ratio of endogenous total (including fecal) nitrogen excretion to basal metabolism is nearly the same (about 2.4 mg. N per Calorie) for mice, rats, pigeons, chickens, and rabbits. Moreover, varying the environmental temperature between the limits of 0° and 30° did not affect this ratio. It appears that the ratio N/Cal. is also constant in poikilotherms, but, unlike the ratio in homoiotherms, is constant only within the species. Thus Bonnet (65) found that changes in environmental temperature (within the limits of 2° and 30° C.) do not affect the N/Cal. ratio in frogs and turtles, but that the ratio was 2.8 for turtles, and 7.2 for frogs. Incidentally these high N/Cal. ratios indicate that cold-blooded animals need a greater proportion of their calories in the form of proteins for purposes of maintenance. It appears from the researches of Terroine *et al.* (66) that when animals are on nitrogen-free diets the excretion of allantoin, urea, amino acids, and ammonia likewise tends to vary with basal metabolism. Smuts (67), in an excellent research carried out in H. H. Mitchell's laboratory, found the following ratios of endogenous urinary nitrogen (in milligrams) to basal metabolism (in Calories): 1.92 for mice, 2.00 for rats, 2.04 for swine. These results confirm the conclusions of Terroine & Sorg-Matter.

Incidentally the constancy of the N/Cal. ratio indicates that the lowest level of nitrogen excretion, like basal metabolism, is reached only at thermal neutrality; it also explains the recent observation by Boothby, Adams & Bollman (68) that on the same food intake an increase in body temperature of a dog by 0.5° F. changed its nitrogen balance from positive to negative. It may also explain the fact noted by Peters & Van Slyke (69) that febrile conditions are accompanied by high blood non-protein nitrogen.

As noted in a preceding section, Brody, Procter & Ashworth (40) found that the basal metabolism in Calories,  $Q$ , and endogenous urinary nitrogen excretion,  $N$ , both vary roughly with the 0.73 power of body weight,  $M$ , as follows:  $Q = 70.5 M^{0.73}$ , and  $N = 146 M^{0.72}$ . The N/Cal. ratio is thus nearly constant, namely  $146/70.5$ , which equals 2.0. This further substantiates the Terroine-Sorg-Matter ratio, but too much importance must not be given to the absolute value of the N/Cal. ratio as indicated by the following results. The power equation  $Y = XM^a$  was fitted to the data of Terroine & Sorg-Matter for endogenous total (including fecal) nitrogen excretion. The resulting equation was  $TN = 283 M^{0.735}$ , where  $TN$  is total nitrogen in

milligrams per day for body weight,  $M$ , in kilograms. On fitting it to endogenous urinary nitrogen data by Terroine *et al.* (70), it was found to be  $Ur.N. = 201 M^{0.720}$ , where  $Ur.N.$  is endogenous urinary nitrogen in milligrams per day for body weight,  $M$ , in kilograms. If we assume, as appears reasonable, that the value of the exponent is the same (within limits of experimental and computational errors) in the four equations above, then Terroine's data on total nitrogen excretion are 40 per cent above ( $283/201 = 1.408$ ) his data on urinary nitrogen; and Terroine's data on urinary nitrogen are 38 per cent above ( $201/146 = 1.38$ ) the data given by the second equation above. If the coefficient, 70.5, in the first equation above is taken as the standard for basal heat production, then the ratio,  $N/Cal.$ , becomes 4.0 ( $= 283/70.5$ ) for Terroine's data on total nitrogen and 2.85 ( $= 201/70.3$ ) for his data on urinary nitrogen. The fact that Terroine & Sorg-Matter found a ratio of 2.4 indicates that their basal metabolism as well as their endogenous nitrogen values happened to be high in comparison with the data included in the first two above.

It is no surprise that data from different laboratories yield different  $N/Cal.$  ratios inasmuch as the level of "endogenous nitrogen" depends on the time during which the animal is kept on the nitrogen-free diet and the level of protein intake preceding the placing of the animal on the nitrogen-free diet. Ashworth & Brody (71) investigated this problem on growing rats with the following results: (a) minimum urinary nitrogen was attained in different individuals any time between four and twenty-eight days on the nitrogen-free diet, and it was therefore concluded that endogenous nitrogen values as obtained by simply keeping rats on nitrogen-free diets for an empirical number of days are not reproducible except on a statistical basis; (b) the lowest  $N/Cal.$  ratio, as determined from the time curve on the nitrogen-free diet, was found to be 1.4 mg. N per Cal. rather than 2.0 as found by Smuts and from the first two equations discussed in the preceding paragraph; (c) the level of protein intake preceding the period of nitrogen starvation has a profound influence on the time required to reach the minimum level of nitrogen excretion (it required, on the average, eight days to reach the minimum level for "low-protein" rats, and twenty-eight days for "high-protein" rats).

*Neutral sulphur excretion.*—Amann & Mourot (72), from Terroine's laboratory, presented extensive data on neutral sulphur excretion and on the relation of neutral sulphur excretion to basal metabolism. Brody, Procter & Ashworth (40) found that these data can be

represented by the equation  $NS = 6.53 M^{0.718}$ , while other data from the literature yield the equation  $NS = 9.64 M^{0.718}$  where  $NS$  represents the excretion of neutral sulphur in milligrams and  $M$  is the body weight in kilograms. This means that the increase of neutral sulphur excretion tends to follow the same courses with increasing body weight as do endogenous nitrogen and basal metabolism. However, variability of neutral sulphur data is much greater than variability of the data on basal metabolism and endogenous nitrogen because, as explained by Amann & Mourot, neutral sulphur excretion is not altogether independent of the diet.

*Creatinine excretion.*—Unlike basal metabolism, endogenous nitrogen, and neutral sulphur, all of which tend to increase with the 0.73 power of body weight, or with surface area, or with weight of visceral organs, preformed creatinine tends to increase directly (linearly) with body weight (40). Ashworth & Brody (71) found that while the ratio of total creatinine to nitrogen remained practically constant in rats between the ages of 20 and 600 days the endogenous urinary nitrogen coefficient declined with increasing weight and therefore the ratio, creatinine nitrogen/endogenous urinary nitrogen, increased with increasing body weight, so that creatinine excretion is not a directly proportional index of endogenous nitrogen excretion, basal metabolism, or neutral sulphur excretion. The equation relating creatinine nitrogen,  $CN$ , with body weight,  $M$ , for mature animals of different species ranging in weight from 0.02 to 400 kg. was found (40) to be  $CN = 12.7 M^{0.898}$ ,  $CN$  being expressed in milligrams and  $M$  in kilograms; but for growing animals of the same species the exponent was very near unity, e.g., for rats,  $CN = 14.6 M^{1.00}$ ; for Holstein cows,  $CN = 9.39 M^{1.02}$ ; for humans,  $CN = 2.4 M^{1.29}$ .

*Endogenous fecal nitrogen.*—One difficulty in evaluating endogenous nitrogen excretion is the variation in fecal nitrogen excretion. Excretion of endogenous fecal nitrogen depends not only on body weight and structure of the digestive tube but, as pointed out by Schneider (73) and by Mitchell (74), also on the nature and amount of (nitrogen-free or nitrogen-low) food fed to the animal while collecting the endogenous nitrogen. According to these authors the metabolic, or endogenous, fecal nitrogen is made up of truly excretory fecal nitrogen, as during fasting, and a digestive waste which varies directly with the intake of dry matter. Schneider (73) found that the fecal nitrogen excretion in the rat is increased by 1 mg., if the dietary dry matter intake, nitrogen-free, is increased by 1.26 gm. An analysis of

Mitchell's data (74) indicates (40) that if the body weight of the rat is held constant, the fecal nitrogen is increased by 1 mg., if the high-carbohydrate diet is increased by 1.31 gm. or if the high-fat diet is increased by 1.43 gm. Similar analysis (40) of data on rats by Boas Fixsen & Jackson (75) indicates that when the body weight is held constant the fecal nitrogen is increased by 1 mg. if the dietary dry matter is increased by 0.52 gm. There are also tremendous species differences with regard to endogenous fecal nitrogen excretion as explained elsewhere (40).

*Possible significance of above facts relating to basal metabolism, endogenous nitrogen, neutral sulphur, and creatinine.*—While basal metabolism, endogenous nitrogen, and neutral sulphur excretion increase with the 0.73 power of the body weight, creatinine excretion increases directly (1.0 power) with body weight. The direct proportionality between creatinine and body weight is in conformity with all the evidence that creatinine represents the muscular mass of the body. The correlation between basal metabolism and endogenous nitrogen in all mature warm-blooded animals indicates a general similarity in the chemistry of animals, a constant level in all animals for the balance between breakdown and resynthesis. Such a balance, and correlation between energy and nitrogen metabolism, might be expected from Borsook & Keighley's theory (45, 76) since (a) deamination is an oxidative process; (b) urea synthesis requires the combustion of some metabolite, whether oxygen is used or not; and (c) resynthesis of ammonia and amino acids to protein requires energy. This much cannot be said as regards neutral sulphur, since it is not a well-defined entity and, as shown by Amann & Mourot (72), the neutral sulphur excretion is not independent of protein intake.

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## THE CHEMISTRY OF MUSCLE\*

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In the construction of this review a choice has been made of a few branches of the subject in which it seemed that progress has been most marked, and the bibliography is restricted to papers dealing with these topics. If the results of the last two years' activity can be summarised in a sentence they point more and more to the intimate connection of phosphate with muscle activity—a belief expressed, it will be remembered, as early as 1921 by the late Gustav Embden.

*Chemical measurements.*—The estimation of lactic acid has received attention in several quarters. The Mendel-Goldschneider colorimetric method has been examined in detail by Nordbö, improved in certain details, and compared with the gasometric method of Avery & Hastings (1927). Kriegsmann has also studied the method and applied the Pulfrich photometer to the colour comparison. Friedemann & Graeser have meanwhile published certain further improvements in the distillation method originally put forward by Clausen. Stewart, Dickson & Gaddie have examined the extent of interference in this method produced by the presence of pyruvic aldehyde. Some interference is to be expected since this aldehyde is volatile in steam, but the interference is enhanced if the protein-free extract is first treated with copper sulphate and lime in the customary way for removal of sugar. The authors find that a distillation of the protein-free extract before copper-lime treatment is the best way of removing the pyruvic aldehyde. Hahn & Niemer have modified their method for the estimation of pyruvic acid, the final stage being the isolation of the crystalline phenylhydrazone.

The determination of reduced glutathione is carried out by Binet & Weller on the basis of precipitation with cadmium (supplied as the lactate) at pH 6 to 7. Cysteine is similarly precipitated at pH 3 to 4.6, and can be separated from reduced glutathione in this way. The estimation of glutathione is also the subject of a paper by Okuda & Ogawa, who titrate the reduced form with potassium iodate, and of several papers by di Capua.

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The Pulfrich photometer has been applied by Bühler to the colorimetry of creatine and creatinine. The estimation of carnosine is effected by Kapeller-Adler & Haas in protein-free extracts of muscle by acid hydrolysis and determination of the liberated histidine by Knoop's reaction. Wilson & Wolff determine anserine in protein-free extracts of muscle by first determining the carnosine (Koessler-Hanke method), then determining the sum of the two, which is given by the increase in amino nitrogen after acid hydrolysis (amino nitrogen by Van Slyke).

The solubility of glycogen in aqueous alcohol has been re-investigated by Somogyi, who puts forward, on the basis of his results, a simple method of isolating the substance in a form free from nitrogen and phosphorus. Brookens (1) has re-examined the heat of combustion and arrived at the figure of 3761 cal. per gm. of hydrated glycogen  $(C_6H_{10}O_5 \cdot H_2O)_n$  in dilute aqueous solution. Taken in conjunction with the accepted value for lactic acid, this gives the figure of 160 cal. for the conversion of glycogen to lactic acid. This heat of glycogenolysis is a figure of great theoretical interest in muscle biochemistry.

*The chemical architecture of muscle tissue.*—The condition of the bicarbonate in muscle has been the subject of three important papers. Brookens (2) finds that sartorii of frogs kept in serum under known pressures of carbon dioxide come to equilibrium with respect to total carbon dioxide content when the muscle contains 70 per cent as much as the serum. In Ringer's solution the difference was much less. Wright has measured the diffusion coefficient of carbon dioxide through living muscle (frog and dog), finding a value of  $6$  to  $7 \times 10^{-4}$  sq. cm. per minute. This value is slightly greater than that for carbon dioxide diffusing through a 20 per cent gelatin jelly. S. L. Cowan has measured the carbon dioxide-dissociation curves of crab nerve and muscle, and from his results calculated the pH of the tissues at different carbon dioxide pressures. He finds an almost linear relation between the  $cH$  and the carbon dioxide pressure over the range 70 to 400 mm. Hg.

Eggleton & Eggleton find that although histidine appears able to diffuse from Ringer's solution into all the water in living frog muscles, carnosine can only diffuse into 30 per cent of it. This makes it likely that the carnosine occurring naturally in the muscles is contained within enclosed regions including 70 per cent of the water, for the naturally occurring carnosine is unable to diffuse out while the muscle

is alive. Cori & Closs record that the concentration of fermentable sugar in the rabbit's heart is approximately one-third that in the plasma, over a wide range of concentrations (60 to 180 mg. per 100 cc.). The simultaneous concentrations in skeletal muscles are even smaller, about 15 per cent of the plasma figure. A similar ratio was observed in fasting rats and in frogs. Conway & Kane have studied the equilibrium between frog muscle and Ringer's solution in respect of sulphate and chloride, and find a concentration ratio of 0.25 to 0.3, respectively; they have also measured the rates of diffusion and find the two anions to have the same diffusion constant,  $6 \times 10^{-5}$ . The equilibrium between frog muscle and Ringer's solution in respect of potassium appears to depend on the hydrogen ion concentration, according to Fenn, and Fenn & Cobb, in such a manner as to keep the product  $[K][OH]$  nearly constant. At pH 6.3, for example, it requires 75 mg. of potassium per 100 cc. to prevent loss of potassium from the muscle (sartorius) while at pH 7.7, 10 mg. per 100 cc. suffices. The optimum concentration for irritability is 25 mg. per 100 cc. at any pH; it is therefore independent of the direction in which potassium is diffusing. (Other papers on the distribution of potassium in muscle, and its variation with age, etc., are those of Ernst & Fricker; Leulier, Bernard & Bérnard; Leulier, Bernard, Bérnard & Richard; and Millard.) M. G. Eggleton (1) has measured the equilibrium conditions between frog muscle and Ringer's solution in respect of phosphate, and finds the behaviour of the system most easily explicable on the view that 70 per cent of the muscle water takes no part in the diffusion system.

Work of this kind will enable us, in time, to form a better idea of the distribution and condition of these substances, though interpretation is at present rather tentative. Closely related to this question is the old problem of the state of water in muscles. Two papers from Brooks (1, 2) have appeared in the last year on this question. This author has measured the vapour-pressure isotherms, by different methods; the originals should be consulted for interpretation.

By the introduction of a capillary glass electrode into the muscles of lightly anaesthetised rats, Voegtlin *et al.* have been able to demonstrate that the normal pH of these muscles is about 7.55 in the resting state. Conditions of oxygen lack caused a shift towards acidity, and death by haemorrhage, asphyxia, cyanide, carbon monoxide, or curare poisoning resulted in the attainment of a pH of 6.7 to 6.9 (extreme value noted was 6.21). Their observations of the effect of iodoacetate

poisoning confirm earlier work on frogs in that there is a drift towards alkalinity (almost to pH 8) finishing in a contracture.

*Activity of isolated muscles.*—Biochemical experiments with isolated intact muscles have been directed chiefly to the elucidation of the time course of the chemical events accompanying a single twitch. It has already been shown that the production of lactic acid, associated with a twitch, occurs mainly, if not entirely, after relaxation, and is coincident with the anaërobic resynthesis of creatinephosphoric acid. Lundsgaard has now shown that in the case of muscles poisoned with iodoacetate and stimulated with a short tetanus in nitrogen at low temperatures (2° C.) a breakdown of creatinephosphoric acid occurs after relaxation is over. This may mean that creatinephosphoric acid breakdown, like lactic acid production, is producing energy for the restitution of some system more directly connected with the contractile mechanism. This system may be a purely physical mechanism (see Ritchie) or it may use energy released by the hydrolysis of adenylypyrophosphate [see Lohmann (3)]. Or we may have to picture a succession of events such as:

*a*—Physical system discharges and energises contraction.

*b*—Adenylypyrophosphate + glycogen → Hexosephosphate + adenylic acid + energy to recharge physical system.

*c*—Phosphagen + adenylic acid → Adenylypyrophosphate + creatine.

*d*—Hexosephosphate + creatine → Phosphagen + lactic acid + inorganic phosphate.

*e*—Lactic acid + oxygen → Glycogen + CO<sub>2</sub> + H<sub>2</sub>O.

This scheme is only a tentative expression of the kind of system which is coming to light as a result of recent research. The shorthand expressions are not strictly balanced chemical equations, but rather abbreviated statements of groups of chemical reactions coupled more or less closely with respect to their energy accompaniments. Further, one must suppose that the relative extents of the successive groups of events may vary according to circumstances (except that *b* and *c* must be closely matched, for no breakdown of adenylypyrophosphate can be observed to result from any reasonable degree of activity).

The work of Meyerhof & Möhle (1, 2, 3), Hartmann, and Ernst & Uj on the time course of the volume changes associated with contraction is another attempt to trace the possible succession of chemical events involved in activity. The measurements

of electrical conductivity during contraction made by Dubouissin and Hartree (1) have been projected with a similar view, but no significant change of resistance has been found to occur (see also Sudo). The paper by Baeyer & Muralt, on changes in translucency of muscles during activity, should also be consulted in this connection.

Meyerhof, Gemmill & Benetato find the oxygen consumption of frog sartorii, relative to the tension-length production in single isometric twitches, after allowing for the resting oxygen consumption, to be approximately  $690 \times 10^6$  (i.e., 6.9 kg.-m. per mg. of oxygen). For muscles poisoned with iodoacetate the slightly lower value of  $670 \times 10^6$  was obtained, but the difficulty of these measurements is so great that this difference is probably not significant. Iodoacetate produces no change in the oxygen-consumption rate of resting muscles (Moede), nor does it prevent oxidation of lactate by active muscles (Mawson). It is becoming more apparent that the relation of lactic acid production to tension-length measurement in isometric activity is affected by a number of factors, and the ratio of one mg. of acid per kg.-m. of tension-length, established a few years ago, must be regarded as a first approximation only. Riesser & Miura give evidence that the lactic acid production is affected by the duration of contraction and not merely by the work done in isotonic twitches. Cori & Cori (1) find in the case of rat gastrocnemii a considerable formation of hexosephosphate during a five-second tetanus, accounting indeed for nearly half the glycogen lost. Using frog gastrocnemii, Fisher & Cori find that the hexosephosphate formation depends upon the rate of activity, being greatest for a tetanus. Lippay & Rand find that stimulation can bring about lactic acid production even in muscles rendered practically inexcitable by the use of Ringer's solutions with a high potassium chloride content.

*Myothermic measurements in relation to biochemistry.*—Of the "initial heat" of an isometric contraction of a frog's sartorius, about one-third is liberated during the onset of tension and two-thirds during its dissipation. None is developed at the moment of maximum tension. A short isometric tetanus differs only in an additional steady production of heat during the maintenance of tension [Hartree (2)]. This maintenance heat-rate depends upon the length to which the muscle is extended (Fenn & Latchford). The heat coefficient ( $Tl/H$ ) for a single twitch is found by Rosen-

berg, using improved methods, to be 30 per cent greater than has previously been thought. His value is approximately 8.25. The delayed anaërobic heat following a single twitch is found by Bugnard to be only 3 per cent of the initial heat and this author confirms Hartree's suspicion that there is a phase of heat absorption during the first twenty seconds following the twitch. Cattell & Parkinson find that the heat coefficient rises during the first dozen or so twitches of a series, then falls to a steady value of 88 per cent of the original. This fall may be due to the delayed heat of the earlier twitches being added to the initial heat of the later ones. A similar change in efficiency is found by Cattell & Lundsgaard for sartorii contracting in oxygen.

The oxidative heat following isometric activity has also received further attention. For single twitches it is found to be 1.06 to 1.1 times the initial heat [Cattell (1, 2)]. Bugnard, however, obtains the much lower value of 0.69. Both authors are agreed that the value rises in a series of twitches or a short tetanus, a finding in agreement with earlier work. Other papers on this subject are from Szabuniewicz and Bouckaert & Capellen (1, 2). Bugnard reviews the interpretation of these facts in the light of chemical evidence and considers that, given an ample supply of oxygen, the muscle obtains its energy for contraction from the hydrolysis of creatinephosphoric acid and resynthesises this acid by means of energy derived directly from oxidation processes without the intermediate formation of lactic acid.

*Other work on isolated muscles.*—The effect on metabolism of passive stretching of frog muscle has attracted attention in several quarters. Weiss records that stretching increases the survival time considerably. Margaria finds that stretching produces an immediate, reversible shift of 0.5 pH towards alkalinity, as judged by the change of colour of muscles previously impregnated with brom thymol blue. Margaria has previously shown that stimulation produces the same effect. He discusses the interpretation of this effect in relation to Feng's observation that stretching increases the oxygen-consumption rate. [See also Euler on the magnitude of the Feng effect in acid and alkaline conditions, and the results of Root (1, 2) on oxygen-consumption rate in relation to partial pressures of carbon dioxide and oxygen.]

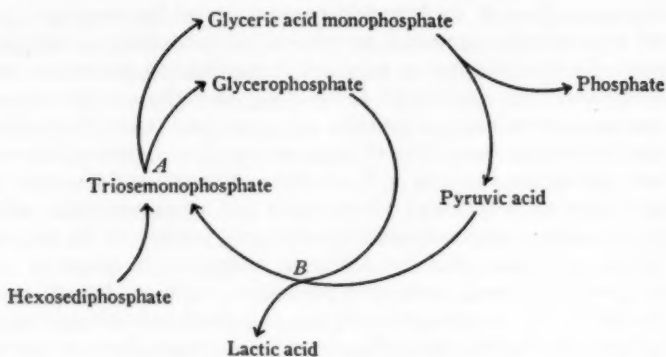
The results of Fenn & Cobb on the equilibrium between frog muscle and Ringer's solution in respect of potassium, to which reference is made elsewhere in this review, form part of a study by



Hegnauer, Fenn & Cobb and Hegnauer (1) of the mechanism of the inexcitability produced by excess of potassium in Ringer's fluid. They record that in solutions containing less potassium than the optimum for irritability (20 to 25 mg. per 100 cc.) the oxygen-consumption rate is independent of potassium concentration, but that above this range the oxygen consumption increases sharply. The membrane potential falls as the potassium concentration of the Ringer's solution rises and has only half its normal value when the potassium concentration of the Ringer's solution is 60 mg. per 100 cc., at which point irritability is completely in abeyance. At this potassium level, lactic acid production begins to be enhanced. Changes in the phosphagen-inorganic phosphate balance also occur, but are less simply related to potassium concentration. A similar study has been made by Hegnauer (2) of the inexcitability produced by substitution of sugar for sodium chloride in Ringer's solutions.

A group of papers from Ronzoni & Kerly (1, 2), Kerly & Ronzoni, and Cori & Cori (3) may be considered together. Frog muscles kept in Ringer's fluid at pH 6 without oxygen do not form lactic acid, but their hexosemonophosphate content increases markedly. At pH 9, on the other hand, lactic acid is produced but no hexosemonophosphate. In muscles first kept anaërobic at pH 6 and then transferred to oxygenated Ringer's fluid at pH 7, the accumulated hexosemonophosphate disappears; there is a temporary enhanced oxygen consumption during which the creatinephosphoric acid content rises again to normal. There is no resynthesis of creatinephosphoric acid in muscles transferred from a Ringer's fluid at pH 6 to one at pH 9 if oxygen is withheld. Hexosemonophosphate is formed as a result of tetanic stimulation, and its subsequent disappearance is shown by Cori & Cori to be more rapid in oxygen than in nitrogen. The anaërobic removal seems to be effected by glycolysis, for it is accompanied by an equivalent increase in lactic acid content and by the liberation of inorganic phosphate.

*Intermediate stages in the formation of lactic acid.*—Early in 1933 it was suggested by Embden, Deuticke & Kraft that the production of lactic acid from glycogen in muscle tissue occurred with the intermediate production of hexosediphosphate (a suggestion made by Embden several years before, but withdrawn in 1926 when he isolated hexosemonophosphate from muscle). Further, the later stages were claimed to be of a cyclic character:

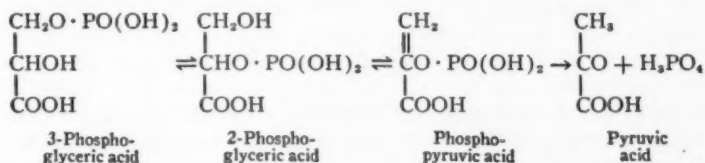


At *A* and *B* interaction occurs between two molecules, resulting in the oxidation of one and the reduction of the other. The following recent reports are in harmony with this view.

Meyerhof & McEachern found that, in the presence of sulphite, pyruvic acid accumulated in muscle extracts during the conversion of glycogen or hexosediphosphate to lactic acid, and the yield of lactic acid was correspondingly diminished. Added lactate did not increase the yield of pyruvic acid. These authors confirmed Embden in finding also an intermediate formation of glycerophosphate, and they showed that although neither of these substances alone was converted into lactic acid, the two together were, and the yield of lactic acid was greater than the amount of pyruvate added; this was confirmed by Boyland & Mawson. If, however, fluoride were present also, the lactic acid formed equalled the pyruvic acid which disappeared. These facts agree well with the scheme above and indicate that fluoride inhibits the breakdown of phosphoglyceric acid (see also Meyerhof). On the other hand bromoacetic acid (and presumably also iodoacetic acid) appears to make impossible the oxidation-reduction processes *A* and *B* (Embden & Deuticke). A later paper of Meyerhof & Lohmann (1) shows that, in a coferment-free muscle extract, hexosediphosphate is converted to lactic acid only in an alkaline reaction. In acid conditions methylglyoxal is the product. This points to dihydroxyacetone monophosphate as an intermediate. This substance can be converted into hexosediphosphate by muscle extracts (coferment-free) and indeed the system proves to be reversible. Dihydroxyacetone monophosphate, synthesised in this way

from hexosediphosphate, proves to be identical with Kiessling's synthetic product, and the hexosediphosphate produced from synthetic dihydroxyacetone monophosphate proves on isolation to be the Harden-Young ester.

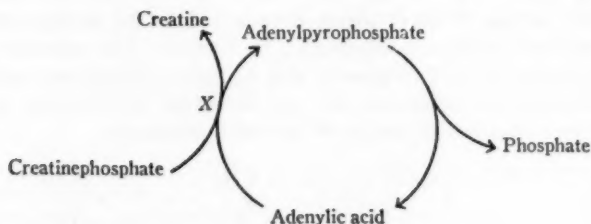
The scheme outlined above does not claim to contain all the intermediate substances which may be formed. The conversion of phosphoglyceric acid to pyruvic acid has been shown very recently by Meyerhof & Lohmann (2) and Meyerhof & Kiessling to involve two intermediate stages of reversible character:



Lohmann (1) has also shown that the conversion of glycogen to lactic acid in muscle extracts requires the presence of adenylypyrophosphate, magnesium, and orthophosphate, and the "ageing" of muscle extracts is primarily due to the slow decomposition of the first of these. All three must be added to a dialysed muscle extract before glycogen can be converted to hexosediphosphate [Lohmann (1)]. Adenylic acid cannot take the place of adenylypyrophosphate but adenylic acid + creatinephosphoric acid are effective [Lohmann (4)], because, as the same author has shown, an enzymic reaction occurs between the two latter substances resulting in the formation of adenylypyrophosphate [Lohmann (3); see also Holmberg].

Adenylypyrophosphate is therefore linked up both with the muscle-glycolysis system and with the creatinephosphoric-acid hydrolysis system, and it is a purely artificial separation if we continue to consider the two apart. Lohmann shows that the hydrolysis of creatinephosphoric acid in muscle extracts cannot occur in the absence of adenylypyrophosphate but that it involves the cyclic formation and decomposition of adenylypyrophosphate, as illustrated on page 422. Thus, at pH 9 the cyclic process fails at X with the result that creatinephosphoric acid is no longer broken down but adenylypyrophosphate disappears (see also Waldschmidt-Leitz & Kohler; Mozolowski & Sobczak; Jacobsen). A number of papers from Parnas and his school deal with this question, though from a different standpoint [Parnas, Ostern & Mann (1, 2, 3); Ostern; Mann-Lutwak].

Side by side with this picture of glycolysis in muscle there has accumulated a body of facts which point strongly to the existence of an alternative mode for the production of lactic acid, involving the



intermediate formation of methylglyoxal. Rapkine (1, 2) showed that iodoacetate reacts with sulphydryl groups in cysteine and glutathione and in denatured proteins with the liberation of iodide. Dickens (1) showed that bromo- and chloroacetic acids could also react with glutathione and measured the velocity constants of the three halogen acids. He showed (2) that the ability of iodoacetate to inhibit glyoxalase preparations could be reversed by the addition of glutathione and that glyoxalase preparations, deactivated by dialysis, were reactivated by addition of glutathione. Jowett & Quastel (1, 2) produced evidence of an intermediate combination of methylglyoxal with glutathione and showed that oxygen deactivates glyoxalase, presumably by oxidising the glutathione. Platt & Schroeder confirmed the formation and existence of the intermediate compound. Mowatt & Stewart report that iodoethyl alcohol like iodoacetate inhibits glycolysis in blood, but, unlike iodoacetate, its inhibitory effect is not combated by glutathione, nor does it react with glutathione *in vitro*.

These facts would provide an explanation of the poisonous action of iodoacetate on muscle, were it not that Lohmann (2) finds that glutathione-free muscle extracts convert glycogen to lactic acid and that addition of glutathione brings about no additional glycolysis. Yet iodoacetate brings about its typical inhibition of lactic acid formation both in the presence and absence of glutathione.

Apart from its effect in inhibiting lactic acid formation it is clear that iodoacetate must exert a rather general poisonous action though it may be only in considerably larger doses. Waldschmidt-Leitz, Scharikova & Schäffner found iodoacetic acid to inhibit nucleotidase and phosphatase. Michlin & Rubel found inhibition of the proteases

of tissue extracts. Michaelis & Schubert record that iodoacetate reacts with amino groups of amino acids at room temperature in faintly alkaline solution. Jacobsen reports inhibition of the synthesis of adenylic acid to adenylypyrophosphate. Barth, on the other hand, finds no interference with diastase, lipase, or pepsin, even from relatively strong solutions of iodoacetate (0.2 per cent).

*Cardiac muscle.*—The heart of the frog is a muscle especially suitable for studying problems concerning anaërobic metabolism, and in the hands of Clark, Gaddie & Stewart it has yielded information of great interest. Following up earlier work previously reported in this *Review*, these authors have shown (1) that the pressure of oxygen available to the surviving heart affects the choice of foodstuffs oxidised, and under an oxygen pressure of one atmosphere the heart consumes less carbohydrate and considerably more of some substance or substances characterised by a low respiratory quotient. Attempts to identify this with fat failed, however. An important advance was made by Gaddie & Stewart who found that the surviving ventricle, deprived of oxygen and bathed with alkaline Ringer's fluid, eventually fails from lack of available carbohydrate, for in this state administration of glucose brings about immediate recovery. Mannose was also effective, but not fructose, nor any of a number of other carbohydrates tested. Methylglyoxal brought about a partial recovery, and so also an equal mixture of pyruvate and glycerophosphate, though neither of these alone was effective. These experiments, therefore, support both the Embden-Meyerhof view of glycolysis and the methylglyoxal school. Further support was afforded to the latter view by the observation that the ventricle, brought to a standstill in nitrogen by addition of iodoacetate to its perfusing fluid, was restored by subsequent administration of glutathione. The authors conclude that glycolysis can occur in this tissue by either of two routes. Further work on asphyxia of the heart is contained in a later paper [Clark, Gaddie & Stewart (2)]. Davis, da Corta & Hastings record that the ultimate failure of the isolated frog heart supplied with oxygen may be due to loss of thyroxine, for hearts perfused with Ringer's fluid containing thyroxine showed, after about fifteen hours, a considerably increased oxygen consumption (experiments lasted forty-two hours), whereas controls steadily declined in oxygen-consumption rate. Two other communications on the oxygen consumption of the frog heart have appeared from Brody and Victor.

A number of papers have appeared dealing with the metabolism of

the mammalian heart-lung preparation [Cruickshank; Cruickshank & Startup (1, 2); Evans, Hsu & Kosaka; Melik-Megrabov; Pollock *et al.*; Rolshoven; and Rühl & Rolshoven]. Perhaps the most important point established is that the heart can take lactate from the blood supplied to it, presumably burning the lactate. Thus Evans, Graff, *et al.* record that the heart (dog) takes up 234 mg. of lactate per hour, and only 7 mg. of glucose. The actual concentration of lactic acid in the venous reservoir may either increase or decrease in the course of an experiment; only the glucose content shows a steady fall. This has led earlier workers to believe that the heart is utilising the glucose directly. It is only when the composition of the blood entering and leaving the coronary circulation is compared (McGinty & Miller; Rolshoven; Rühl & Rolshoven; Evans, Graff, *et al.*) that it becomes evident that the changes in the venous reservoir are the resultant of two successive changes: glycolysis, partly spontaneous, partly induced in the lungs (Evans, Hsu & Kosaka) and absorption of lactate by the heart. The latter is inhibited by cyanide, according to Rühl & Rolshoven, and slightly increased by iodoacetate (Evans, Graff, *et al.*). Other papers concerned with the carbohydrate metabolism of the heart are: Tanzi; Cori, Closs & Cori (distribution of fermentable sugar between cardiac muscle and blood plasma); Blume (glycogen content of human heart) and Evans (effect of various conditions on glycogen content of rat heart). Creatinephosphoric acid changes have been studied by Weicker (cat and dog) and Mügge; the creatine content of human hearts (left and right ventricle compared) is reported upon by Seecof, Linegar & Myers; and of rat hearts (experimental hypertrophy) by G. W. Cowan.

The nucleotides of cardiac muscle have received attention from Beattie, Milroy & Strain and Strain, from chemical, physiological, and pharmacological standpoints. Richards has recorded a study of the effect of muscle nucleotides on a human heart and blood vessels. Winter has published a study of the condition of the inositol in the heart (dog).

*Exercise in man.*—Except for Wilson's measurements of sulphur and nitrogen excretion after severe exercise on diets of different protein contents, the biochemical study of exercise in man during the period under review has been largely concerned with respiratory measurements and the phosphate and lactate levels of the blood. Kalk & Bonis found no change in the ammonia concentration of the blood in the cubital vein during and after vigorous exercise of the fore-

finger. Small increases in blood phosphate following certain kinds of exercise continue to be recorded. Planet & Cardoso found both creatine and inorganic phosphate to rise. Gemmill & Ribeiro find, however, that a ten-second spell on the treadmill, while not accompanied by a rise in blood inorganic phosphate, is followed during recovery by a slight fall. Experiments with dogs gave the same result.

The observation of Stacey that creatine given by mouth causes a slight disappearance of phosphate from the blood, taken together with the fact that such creatine is temporarily stored in the muscle (an old observation of Folin's), suggests that the creatinephosphoric acid content of the muscles may be temporarily increased.

The pH of the blood was found by Margaria & Talenti to fall as much as 0.23 unit after brief violent exercise, although hard work, not involving oxygen debts, produced no significant change (observed also by Cook & Hurst). The peak of the acidosis is reached only three to six minutes after the exercise (440 yards run) according to Laug. The authors agree that the appearance of lactic acid is a sufficient explanation of the pH change. Laug's object was to correlate lactic acid, alkali reserve, and pH of the blood during the first fifteen minutes of recovery. He observed that the alkali reserve continued to decrease during the disappearance of lactic acid and simultaneous rise of pH.

The interpretation of respiratory exchanges during recovery continues to become less simple than used to be supposed. Solandt & Ridout observe that after thirty to forty-five seconds of severe exercise involving a 5 to 10-litre oxygen-debt, the carbon dioxide elimination rate was normal again in one and one-half hours, but the oxygen-consumption rate had not returned to normal even in two hours. The excess respiratory quotient was always greater than unity. The meaning of this is not clear since extra oxygen was still being taken in when the experiments were terminated. It is possible that this persistent increase in metabolic rate is the result of a stimulation of the thyroid gland, for Herxheimer, Mishowitz & Stanoyevitch record an increased iodine content of the blood lasting for some hours after the conclusion of such exercise. No close correlation could be observed by Bang between lactic acid disappearance from blood and oxygen utilisation after various degrees of exercise involving oxygen debt. The whole subject is clearly complicated by the state of training of the subject, a factor studied by Vladimirov, Dimitriev & Urinson



(1, 2). A very definite advance, however, has been made by Edwards, Margaria & Dill (1) who have shown that the disappearance of lactate from the blood during recovery follows, except during the first few minutes, a course such that the excess lactate,  $y$ , in the blood at any time,  $t$ , after the exercise, is given by  $\log y = a - bt$ . The truth of this relationship was demonstrated not only in the normal subject, but also after bicarbonate ingestion and during respiration in an atmosphere of 40 per cent oxygen (both of which incidentally increased the subject's exercise tolerance) and also during respiration in an atmosphere containing only 13.5 per cent oxygen (which approximately halved the subject's ability). The subject was in all cases running at 11.6 miles per hour on an uphill gradient of 1 in 40. The question as to how far the blood changes are representative of the body as a whole was settled by experiments with mice [Margaria & Edwards (1)] in which the whole animal was analysed at various times after a short spell of severe exercise. It was found that, except for the first few minutes of recovery, the disappearance of lactate from the body followed the relation,  $\log y = a - bt$ , and the velocity constant proved to be of the same magnitude as that calculated from blood analyses in the human subject. As in man, there was observed no significant lactic acid removal during the first three to four minutes of recovery.

A further point of great interest (Margaria, Edwards & Dill) is that no extra lactic acid appears in the blood at all during or after exercise involving oxygen debts of less than 2.5 litres. At metabolic rates greater than this, lactic acid accumulates in the blood to an extent proportional to the additional oxygen debt (7 gm. per litre). These authors conclude that the oxygen taken in during recovery from exercise can be divided into three categories:

(a) Oxygen required for basal metabolism (which may be higher for some hours than the pre-exercise basal rate).

(b) Oxygen required for the removal of an "alactic" oxygen debt, the removal of which takes precedence and is half completed in thirty seconds.

(c) Oxygen required for the oxidative removal of lactic acid, a slow process, fifteen times slower than the removal of the "alactic" debt.

The fact that oxygen debts smaller than 2.5 litres appear to be wholly of the "alactic," rapidly paid off type, suggests to the authors that the anaërobic source of energy used for such degrees of exercise

may be merely the breakdown of creatinephosphoric acid. There is certainly enough creatinephosphoric acid in the muscles to permit such a view and there is no obvious reason why the energy required to resynthesise this should not be obtained directly by the oxidation of any available foodstuff [see also Margaria & Edwards (2)].

The same group of authors have also studied further the question of the utilisation of fat in muscular exercise (Dill, Jones & Edwards). A fasting dog did twenty-seven hours of work on a treadmill and the total work performed, 190,000 kg.-m., was ten times as great as could be accounted for by the glycogen reserves, assuming 10 per cent of glycogen in the liver and 1 per cent in the musculature. In a similar experiment on an athletic human subject, respiratory measurements were also made. The respiratory quotient fell steadily from 0.83 to 0.75, and showed no tendency to change during the intermediate rest periods. In further work of the same kind [Edwards, Margaria & Dill (2)] it was shown that, in exercise of sufficient duration and intensity to reduce the carbohydrate reserves to very low levels, the respiratory-quotient measurements indicated a steadily increasing use of fat, from 8 per cent of the energy supply up to 77 per cent. Alteration of the rate of work at any stage altered the proportion of energy derived from carbohydrate, although the blood-sugar concentration was not affected. Gemmill observed no rise in the acetone bodies of the blood after exercise in men of normal nutrition, but a rise in the blood acetone during exercise of men on a diet poor in carbohydrate, and a further increase which continued for two hours after the conclusion of the exercise.

Other papers bearing on the chemistry of muscular exercise in man will be found in the bibliography under the following references: Nyman & Palmlöv (effect of exercise on rate of oxidation of alcohol); Kahn & Milodivova (oxygen-utilisation coefficient during recovery); Crowden; Garry & Wishart; Hill; Ogasawara; and Wishart (studies of mechanical efficiency).

*Adrenaline and muscle function.*—Adrenalectomy produces no change in the partition of phosphorus in the muscles of cats even when, after five to eight days, gross symptoms of adrenal insufficiency have developed (Lundsgaard & Wilson). The production of lactic acid relative to tension development in isometric twitches is normal in the case of cats (Cope *et al.*) but diminished considerably in guinea pigs (Nachmannsohn). The discrepancy may be due to differences in the degree of adrenal insufficiency. Cope *et al.* find a diminution

in creatinephosphoric acid breakdown per unit of activity [see also Daoud & Gohar (adrenaline and insulin on muscle glycogen); and Cori & Cori (2) (adrenalectomy in rabbits); and Bozler, who finds adrenaline (1:2,000,000) increases the resting heat rate in nitrogen of frog sartorii, an effect also produced by sympathetic stimulation; other communications bearing on this subject are those of Hegnauer & Cori, and Corkill, Marks & Soskin].

*Muscular dystrophy.*<sup>1</sup>—A number of papers have appeared, reporting the effects of administration of glycine to patients with neuromuscular atrophy (Beard & Tripoli), chronic myositis (Reinhold *et al.*), and progressive muscular dystrophy (Reinhold *et al.*; Kostakow & Slauck; Mader *et al.*; Schoo & Boer; Beard & Tripoli; Chanutin *et al.*; Brand & Harris). It seems that administration of glycine produces a temporary improvement (three to four weeks), judged clinically, in cases of progressive muscular dystrophy, but no lasting benefit. Creatine excretion is, in general, increased. It nevertheless seems that there can be no serious shortage of glycine in the bodies of patients suffering from this disease, for Freiberg & West and Shorr, Richardson & Wolff find the ability to excrete hippuric acid in response to ingestion of benzoate to be normal. This was also true of cases of pseudohypertrophic dystrophy and Graves's disease, although the one case of myasthenia gravis examined by Shorr *et al.* showed diminished response. This latter observation accords with the experience of Boothby that glycine therapy produces a marked and more lasting improvement in patients with myasthenia gravis (see also Schmitt).

*Comparative studies.*—Needham's work on the development of the chick embryo has been extended [Baldwin & Needham (1)] by a study of the appearance of creatinephosphoric acid in the developing chick. Creatinephosphoric acid is already present at the seventieth hour of incubation; earlier stages were not studied. These authors have also shown (2) that the muscles of the flies, *Calliphora* and *Lucilia*, contain argininephosphoric acid and no creatine, there being present also adenylypyrophosphate and hexosemonophosphate.

Arnold & Luck have estimated the nonprotein arginine in the muscles of a number of vertebrates and invertebrates. An important point in their results is that muscles of the rat and rabbit contain small amounts of arginine, thus forming exceptions to the rule that

<sup>1</sup> Cf. also this volume, p. 247. (EDITOR.)

free arginine is found only in the muscles of invertebrates. Broude (1, 2) finds arginine, but no carnosine, in muscles of river crabs. Lohmann & Schuster have isolated adenylypyrophosphate from the muscles of lobster, frog, and rabbit. Namiki and Kernot & Speer have examined the phosphagen content of the muscles of a variety of fish. Borsuk, Verzbinskaja & Kreps have followed the changes in the phosphagen content induced by stimulation of the muscles of some ascidians and annelids. M. G. Eggleton (2) has identified the phosphagen of the foot muscle of *Mytilus edulis* as argininephosphoric acid and reports a chemical study of the effects of fatigue and recovery in that muscle.

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## THE METABOLISM OF BRAIN AND NERVE\*

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A good deal of new work on the metabolism of nerve has appeared during 1934. That of Hill (which has been mentioned in previous volumes of the *Review*) has led him to the view that the actual transmission of the nervous impulse is probably unaccompanied by chemical activity, for the initial heat of nerve, measured by the refined methods now available, is so small as to make it unlikely that it arises from a chemical reaction. Hill is inclined to regard it rather as arising from a condenser discharge (Hill, 1932). Such a discharge, however, taking place in a fluid medium, must involve the movement of ions, and one of Hill's pupils, Cowan, has followed up earlier suggestions that the ions concerned may be potassium ions. Using the non-medullated nerves of *Maia*, he has elicited the following facts: (a) *Maia* blood serum contains (on the average) 0.0398 gm. of potassium per 100 gm. of blood (sea water contains 0.0387 gm. per 100 gm.) (b) *Maia* nerve contains 0.5067 gm. of potassium per 100 gm. The ratio  $[K \text{ in nerve}]/[K \text{ in blood}]$  is thus 12.71. (c) Little or no potassium leaks from a nerve resting in oxygenated sea water, but it does leak rapidly if the nerve is stimulated, or is deprived of oxygen. (d) Both the injury potential and the action current are depressed if the concentration of potassium bathing the nerve is increased: there is a linear relation between the logarithm of the outside potassium concentration and depression of injury potential, until the latter has fallen to about 10 per cent of its original value. For total abolition of the injury potential, however, the outside concentration of potassium must considerably exceed that inside the nerve. (e) Dehydration of the nerve (which raises the inside potassium concentration) increases the injury current. (f) Nerves which have become inexcitable can be restored by washing in sea water, the function of which is almost certainly to wash away potassium which has accumulated on the surface. Similarly, nerves which have been inactivated by prolonged exposure to nitrogen are restored only by oxygen plus washing.

The magnitude of the measured injury potential is never more than half the maximum possible, as calculated from the known con-

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centrations of potassium inside and outside the nerve. The magnitude of the injury potential as measured, however, must be seriously decreased by short-circuiting through the tissue. All this seems to point to the likelihood that the nerve impulse depends on the movement of potassium ions, which pass through the membrane during activity either because its permeability is reversibly altered, or because other diffusible ions are produced. Fenn, Cobb, Hegnauer & Marsh performed experiments on the electrolyte content of medullated (frog) nerve. They conclude that the nerve may be considered as containing a "sodium chloride space" and a "potassium space": the "sodium chloride space" is probably in equilibrium with the fluid in which the nerve is bathed; the "potassium space" is not. The latter probably represents the nerve fibres, the former, other elements of the nerve trunk. Each space occupies about half of the nerve. This conception explains the fact that potassium appears to diffuse into the nerve against a higher inside concentration: the potassium is, in fact, entering the "sodium chloride space" where the potassium concentration is low. The nerves of spring frogs contain potassium in a concentration of 4.8 meq. per 100 gm. of nerve, those of autumn ones 3.02 meq. per 100 gm. of nerve. Potassium appears to be less diffusible than other ions, and is presumably held in the fibres. With regard to crab nerve, they find that 25 per cent of the nerve contains sodium and chloride in a concentration equal to that of the surrounding fluid. The remainder contains most of the potassium; the concentration is twelve to fifteen times as great as that outside the nerve. These findings for crab nerve are in general agreement with those of Cowan.

Ets & Boyd find that when a frog nerve is exposed to cold, "blocking" occurs at a higher temperature than usual, if the nerve has previously been soaked in Ringer's solution with a high content of potassium (56 mg. per cent or more). Cocaine (0.1 per cent) and urethane (2 per cent) have an action similar to that of potassium.

The papers just discussed all indicate that the potassium content of the nerve fibre is high, and that the potassium ion does not readily diffuse out of the resting nerve. From active crab nerve it can diffuse, and obviously this fact could readily be explained if activity were accompanied by the production of some other ion, which, itself diffusible, would enable the potassium to pass out with it.<sup>1</sup>

<sup>1</sup> For other work on the diffusion of potassium, cf. this volume, p. 5. (EDITOR.)

Ashford & Dixon have observed a remarkable effect of potassium ions upon the production of lactic acid from glucose by rabbit-brain slices. The presence of 0.1 *M* KCl greatly increases the production of lactic acid in the presence of oxygen, but decreases it in the presence of nitrogen. The first of these effects is reversible, the second is not. The increased aerobic glycolysis is inhibited by *dl*-glyceric aldehyde. Potassium does not affect the oxidative removal of lactic acid by brain tissue; indeed, the total oxygen uptake of the tissue is increased in the presence of 0.1 *M* KCl.

It is difficult, at present, to trace a connection between these potassium effects on nerve and on the brain cortex, respectively, but there is no doubt that both sets of phenomena should be borne in mind. For that reason, the experiments of Ashford & Dixon have been mentioned here, rather than in the discussion of the work on brain, to which they more properly belong. Pichler claims that there is more alcohol-soluble potassium in the central nervous system of the narcotized frog than in that of the normal frog. His results are too variable and his experiments too few to carry much conviction.

Experiments, somewhat parallel to those of Fenn and his collaborators on the potassium content of frog, crab, and lobster nerve, have been made by Tipton on the calcium content of these tissues. He finds that the calcium content is about 0.735 meq. per 100 gm. of fresh nerve in the case of the frog and 1.4 meq. in the case of the crab and lobster. Of this calcium, some 40 per cent will diffuse out into calcium-free Ringer. This suggests that the calcium is probably contained in the intercellular spaces of the nerve—the “sodium chloride space” referred to by Fenn and his colleagues. It is, however, worth remembering that in any case only about 40 per cent of the serum calcium is diffusible through a collodion membrane.

The phenomenon of “cold block” of nerve has received some attention. Boyd & Ets find that freshly excised frog nerves conduct at low temperatures until frozen. “Freezing block” may occur at  $-6^{\circ}$  to  $-1^{\circ}$  C. If freezing occurs without supercooling, recovery is rapid, but if freezing occurs at low temperatures, recovery is delayed and may not occur until the nerve has been soaked in Ringer’s solution. Similarly, repeated blocking and recovery may result in a rise in blocking temperature and in non-irritability, which can only be removed by soaking in Ringer’s solution.

Bamia & Ets find that the nerves of frogs acclimatized to a temperature of  $5^{\circ}$  to  $8^{\circ}$  C. can be cooled to between  $-4^{\circ}$  and  $-9^{\circ}$  C.

without blocking. Block appears at a higher temperature if nerves are treated with an excess of potassium ions and at a lower one if they are treated with excess of calcium ions, so that in the latter case block occurs only on freezing, and after considerable supercooling. The blocking temperature is not much affected by the pH of the surrounding fluid.

Schmitt & Wade have endeavoured to correlate with one another the effects of heat on different aspects of nerve function (frog and lobster nerve). They find that shortening, which is presumably due to coagulation of protein, starts in the case of frog nerve at 48° C. and is complete at 60° C. and in the case of lobster nerve starts at 60° and is complete at 90°. Conduction fails at about 45°, at which temperature, also, respiration falls to about 20 per cent of the normal. The dehydrogenase systems fail at about 45°, and the indophenol oxidase at 47° to 50°. Less information is gained from these experiments than might have been hoped for, since failure in all directions seems to manifest itself at or about the same temperature. Schmitt & Scott have studied the effect of carbon monoxide on the respiration of various tissues, including nerve. A mixture of 79 per cent carbon monoxide with 21 per cent oxygen produces little inhibition of the oxygen consumption of nerve; definite inhibition (in the dark) is produced by 90.5 per cent carbon monoxide plus 9.5 per cent oxygen. The effect is reversed by illumination; indeed, illumination produces an acceleration (above normal) of oxygen consumption, which may be interpreted as the repayment of an "oxygen debt" contracted during the period of exposure to carbon monoxide in darkness.

Schmitt, Skow & Bueker have investigated the effect of arsenite on nerve. They find that 0.0001 *M* arsenite inhibits nerve respiration, reversibly, to the extent of 40 per cent of its normal size, while 0.001 *M* produces an irreversible inhibition. A concentration of 0.0001 *M* arsenite abolishes the action potential, but not until a period of one to three hours has elapsed, by which time the full inhibitory effect on respiration has developed.

In a later communication, Schmitt & Skow (1) show that "nerve oxidase" (*sic*) is accelerated by arsenite, but that the dehydrogenase systems are "blocked."

An earlier paper by the same authors (2) reports that the catalase content of nerve is extremely low ("catalase quotient," 35). Nerve catalase is very sensitive to sodium cyanide and to sodium azide, while the effect upon it of carbon monoxide varies with the period of ex-

posure, short exposures inhibiting, and long exposures accelerating its action. This carbon-monoxide effect is insensitive to light. Catalase action is not impaired by the asphyxiation of nerve, so that even were hydrogen peroxide formed during asphyxia, it would be at once decomposed. Nerve contains a thermostable compound which has a marked catalytic effect on the oxidation of unsaturated fatty acids. While this may be a haem compound, the effect is too marked to be the result solely of the small amount of catalase present in the nerve.

Cohen, Gerard & Tupikow have investigated the effect of iodoacetic acid and of glutathione on nerve and muscle. Both substances decrease nerve respiration, iodoacetate to one-fifth, glutathione to one-half, and a mixture of both to three-fifths, in a space of ten hours. Iodoacetic acid is very slowly inactivated by glutathione. Glutathione prevents the breakdown of phosphocreatine in the presence of iodoacetate, but not that of pyrophosphate. Iodoacetate reduces the nerve action potentials in two hours, glutathione, curiously enough, in twenty minutes. The last recorded fact seems very extraordinary.

Mention was made in the last *Review* of the experiments of Hill and others on the greatly increased heat production and prolonged action potential which appears in nerve which has been treated with veratrine. Schmitt, Graham & Schmitt have investigated again the electrical response of veratrinized nerve, using the cathode-ray oscillograph. They find that, in veratrinized nerve without asphyxia, the "after potentials" last from ten to thirty seconds. If the nerve is veratrinized, asphyxiated, and allowed to recover the "after potentials" may last as long as six minutes. They have also determined the rate of oxygen consumption of veratrinized nerve. The excess oxygen consumption is at a maximum at from ten to thirty minutes after the veratrine has been added to the preparation, and preliminary asphyxiation does not increase the speed with which the effect appears. They conclude that veratrine penetrates the nerve fairly quickly. The magnitude of the increase in oxygen consumption is 1 per cent to 3 per cent at a stimulation rate of sixteen to thirty shocks per minute with or without previous asphyxiation of the nerve. Since Hill found a thirty-fold increase in the heat production at a stimulation rate of thirty per minute and a thousand-fold increase for a single shock, the authors suggest that the increases in heat production and in oxygen consumption may bear no, or only a partial, relationship to one another. In spite of this it is clear that the work on veratrine has served



to correlate, as never before, the three chief aspects of nerve activity: heat production, respiration, and action current. This, and other important points, were emphasized in the discussion on nerve activity held in Chicago in 1934, of which an account will be found in *Science*,<sup>2</sup> to which the reader's attention is especially directed.

Several papers have appeared from Hill's laboratory, dealing with the thermal and electrical aspects of nerve activity; the latter should receive at least brief mention here, though they are hardly within the scope of this review. Hill has brought forward further evidence emphasizing the fact that the heat production observed to occur when nerve is stimulated artificially cannot be an artefact due to local changes set up by the stimulation, as claimed by Winterstein. With the object of demonstrating parallelism between nerve heat-production and other known characteristics of frog-nerve response, he has developed a method whereby the heat production can be compared with the excitation time. This enables the heat production, which we presume to be a measure of chemical activity, to be directly related to the time-intensity factor of the stimulus, already familiar as a characteristic of nerve activity when examined in conjunction with the muscle response. Hill gives curves which relate (a) the heat response to the time of discharge of the condenser in the stimulating circuit, the number of stimuli per second remaining constant throughout and the voltage being constant for each curve, and (b) curves relating the time of discharge of the condenser to the voltage, the heat production being constant. For technical reasons, it was difficult to determine the "chronaxie," so that the excitation time for the stimulus of minimum energy was employed instead, and was found to be constant. This quantity was also constant when mechanical response, instead of heat production, was taken as the "indicator."

Scott has determined the strength-duration (i.e., potential—capacity  $\times$  resistance) curves for frog nerve, using the electric response of the nerve as an indicator. He finds that for short discharge times, the quantity of electricity for a constant degree of excitation becomes constant, and is independent of the time of discharge. While the frequency of stimulation has little effect on the time relations of excitation, steady activity quickens the excitatory process. Feng & Hill, in 1933, investigated the behavior of frog nerve, as judged by its heat production during prolonged activity. They found that, at relatively

<sup>2</sup> *Science*, 79 (Suppl.), April (1934).

low rates of stimulation, the heat production may reach a maximum, and remain there for very long periods; at high stimulation rates, such a "steady state" is not attained. Hill discussed the matter further at Chicago<sup>a</sup> and Bugnard has described additional experiments bearing on the problem. He finds that if a nerve is producing heat at a steady rate in response to a stimulus of 100 per second, or less, and a stimulus of higher frequency is imposed, the heat production immediately decreases. The decrease is greater, the higher the frequency of the second stimulus, and the longer the previous "steady state" has been maintained. Had the high-frequency stimulus been applied to the nerve in a state of rest, a rate of heat production much higher than that of the "steady state" would have resulted, though it would not have been maintained over a long period. All degrees between complete inhibition and steady heat production can be observed, if the frequency of the second stimulus is varied. The phenomena are reminiscent of "Wedensky inhibition," with which, however, the myoneural junction, not the nerve fibre, is supposed to be concerned. This work is difficult to interpret at present. Presumably the duration of, or recovery from, the refractory phase is modified by prolonged activity.

Gerard & Hartline have evolved a most elegant technic for the measurement of the oxygen consumption of small pieces of tissue. With its aid, they have performed experiments which seem finally to settle the question as to whether the increased oxygen consumption which accompanies electrical stimulation of a nerve is a true physiological process. Using the isolated optic nerves and eyes of *Limulus polyphemus*, they have shown that both electrical stimulation and illumination of the retina are accompanied by an increased oxygen consumption, and that the increases in both cases are of the same order of magnitude and have similar time relations. The excess respiration on stimulation is about 40 per cent of the resting respiration, and the period of increased respiration outlasts the period of conduction by fifteen to forty minutes. As well as absorption of oxygen, gas evolution occurs, the gas being, in all probability, ammonia.

Cowan has determined the carbon-dioxide dissociation curve for crab (*Maia*) nerve and muscle. Cowan & Ing have investigated the action of curarine chloride and strychnine metho salts on the frog muscle-nerve preparation.

<sup>a</sup> *Science*, 79 (Suppl.), April (1934).

The biochemistry of brain has been the subject of certain investigations during the period under review. Cohen & Gerard have cytolysed rabbit brain with distilled water and centrifuged the material so obtained. The resulting homogeneous sol was free from cells, and contained little debris; yet they found that it had a measurable respiration, the  $Q_{O_2}$  being of the order of 0.5<sup>4</sup> ( $Q_{O_2}$  of slices of rabbit cortex = 6 to 7). This respiration lasted for four to five hours at 37°, and had an R.Q. of 0.5. The preparation dehydrogenated succinate, paraphenylenediamine, methyl glyoxal, and, to some extent, glycerophosphate. It did not dehydrogenate glucose, fructose, lactate, or pyruvate. It is of great interest that the enzymes responsible for the dehydrogenation of these different substrates have thus been separated in brain.

An inhibition of 30 to 50 per cent occurred if oleate or citrate was added to the preparation. The respiration with succinate, paraphenylenediamine, and methyl glyoxal was inhibited by cyanide, the cyanide effect on the succinate being reversed by methylene blue. The respiration of the preparation with no additional substrate was actually increased by cyanide. Isoelectric precipitation at pH 4.6 gave a precipitate, which, after it had been washed and re-dissolved, respired from one-quarter to one-third as rapidly as the original preparation; but when succinate or paraphenylenediamine was added, the respiration was nearly as great as that of the original material.

Quastel & Wheatley have found that the inhibition of the oxygen consumption of brain tissue in the presence of glucose, which is brought about by narcotics, is very nearly completely reversible if the tissue is washed in glucose-phosphate Ringer after having been shaken in a manometer for one-half to two hours in the presence of the narcotic. For this work, brain slices must be used, since the oxygen uptake of chopped brain is much decreased by washing. The inhibition by hyoscyne, mescaline, and  $\beta$ -phenylethylamine is also reversible, while that brought about by indole is not. None of the substances investigated depresses the oxygen consumption of brain in the presence of succinate, and this is of particular interest in view of the separation of the succinoxidase from the system oxidizing lactic acid by Cohen & Gerard. Quastel, however, advances the argument (in reply to a criticism of an earlier paper of his by Bülow) that, because the succinoxidase system was intact, the substance in question, acetylene, did not act as a general cell poison. In view of the work of Cohen &

<sup>4</sup>  $Q_{O_2}$  = oxygen consumption in cmm. per mg. dry weight per hour.

Gerard, this argument is obviously untenable. Waelsch finds that saline extracts of the brains of anaesthetized animals reduce methylene blue more quickly, both in the presence and in the absence of the substrates, succinate and lactate, than do extracts of the brains of normal animals. He finds that the effect is not due to the hyperglycaemia accompanying anaesthesia, which would have increased the substrate (glucose) content of the preparation. He also reports similar findings for the brains of hens in a state of hypnosis. It is to be hoped that his experiments will be published elsewhere in fuller detail: the details given in the present paper are too few to permit a proper assessment of the work.

Ashford has examined further the glycolytic mechanism of brain tissue. He finds that the anaërobic glycolytic rate is far higher with sliced than with chopped tissue, and in bicarbonate /CO<sub>2</sub> buffer than in phosphate buffer. Anaërobic glycolysis is lowered by potassium salts (aërobic glycolysis is increased: see above). Inorganic phosphate is liberated by sliced brain tissue under anaërobic conditions; the rate of liberation is somewhat decreased by glucose, but is not affected by fluoride, although lactic acid formation is very sensitive to fluoride. These latter findings are similar to those of Ashford & Holmes, and are interpreted as supporting their contention that glycolysis in brain does not involve esterification with phosphate. Holmes (unpublished) finds that anaërobic glycolysis of brain, which is very sensitive to *dl*-glyceric aldehyde, is not inhibited by glyceric-aldehyde-phosphoric acid.

Peters and his co-workers have published a series of papers on the effect of vitamin B on pigeon brain. It will be remembered that this group of workers have shown that in vitamin-B deficiency the oxygen consumption of minced brain in the presence of lactate is diminished. The rate of oxygen consumption can be restored by adding vitamin-B<sub>1</sub> concentrates to the preparations in the manometer cups. This effect is referred to as the *catatorulin* effect. The reasons for the use of this term are discussed by Passmore, Peters & Sinclair. Peters & Sinclair find this effect to be maximal at pH 7.3; it is abolished by cyanide and fluoride; it is improved by sodium pyrophosphate, and at pH 6.6 pyrophosphate is essential for its manifestation. Hexose diphosphate, Robinson's hexose monophosphate, and  $\alpha$ -glycerophosphate all increase the oxygen uptake of normal and avitaminous brains. The authors found a positive reaction for pyruvate when avitaminous brain was shaken aërobically with lactate, and they observed that

pyruvate showed some catatorulin effect. Sinclair finds that the R.Q. of the brains of polyneuritic (avitaminous) pigeons is low, and that it is raised by the addition of the vitamin *in vitro*. In the presence of lactate, the R.Q. of the extra respiration, due to the addition of vitamin, is considerably greater than unity.

Peters & Thompson have identified and estimated the pyruvic acid which appears in the avitaminous brain during aërobiosis *in vitro*. They find that its production is depressed by vitamin B<sub>1</sub>, that more appears in the presence, than in the absence, of lactate, and that it also appears in normal brains in the presence of lactate plus iodoacetate, but not in the presence of lactate plus fluoride. Pyruvic acid disappears when shaken with the brain tissue in the absence of lactate. Approximately two molecules of extra oxygen are taken up for every molecule of pyruvate disappearing, but this relationship is very variable. Peters, Rydin & Thompson suggest that vitamin-B<sub>1</sub> acts on some unknown substance in the tissue, which then reacts with pyruvate or lactate, giving the increased oxygen consumption.

Galvão & Florence have investigated the methylene-blue reduction times of the brains of vitamin-B deficient fowls. They find that the shortening of the reduction time which normally occurs when lactate is added to the preparation is diminished (i.e., power of dehydrogenation is decreased) in the case of vitamin-deficient birds. This effect is particularly prominent in extracts made from the optic lobes. No change was seen in the reduction time in the presence of succinate. Jowett & Quastel have investigated the glyoxalase activity of many tissues, among them brain. There appears to be no competitive inhibition of lactic acid formation from glucose by methyl glyoxal. Boyland & Boyland find that 1,2,5,6-dibenzanthracene inhibits both respiration and glycolysis of brain and of other tissues, normal and malignant. Dickens has shown that treatment of brain tissue with phenylhydrazine raises its power of aërobic glycolysis, and depresses its oxygen consumption in the presence of glucose or of lactate. This effect may be contrasted with the effect of potassium, observed by Ashford & Dixon.

Elliott finds that there is but a trace of peroxidase activity in brain tissue, and no catalase activity.

Bamberger & Never have found that the venous blood from the human brain contains less oxygen and more carbon dioxide than the venous blood in any other part of the body. The subjects were infants, and the samples were drawn through the fontanelles.

Edlbacher, Goldschmitt & Schläppi investigated the enzymes of dried brain preparations. They found both the catheptic and the tryptic power to be very small; but phosphate was split off from magnesium hexose diphosphate, nucleic acid, and sodium glycerophosphate. Lasnitzki states that removal of potassium, or calcium, or of both ions from Ringer's solution in which the brain slices are suspended diminishes the anaërobic glycolysis of rat brain.

Riebeling has investigated the ammonia content both of brain tissue and of cerebrospinal fluid. The average value for the cerebrospinal fluid was 0.07 mg. per 100 cc. and it increased on incubation. The initial value was enormously increased in a case of *status epilepticus*. The author confirmed previous findings that the ammonia content of brain tissue (both rabbit and human) increased largely on incubation in bicarbonate solution. In the case of *status epilepticus*, the initial value was 8.0 mg. and the final, 20 mg. per 100 gm. of brain, the figures for normals being 4.0 mg. and 10.0 mg., respectively. The author presents evidence that at least the major part of the ammonia comes from adenylic acid.

Plaut & Bülow have investigated the vitamin-C content of the brain and cerebrospinal fluid in men, rabbits, and mice. They find that the amount of ascorbic acid in both decreases with age. The quantity in the adult rabbit brain is 0.20 mg. per gm. In man it varies from 0.31 mg. per gm. in the brain of a five weeks' foetus to 0.05 mg. per gm. in that of a man aged ninety years. Ascorbic acid is present in the cerebrospinal fluid in a concentration only about one-tenth of that in the brain. It shows, however, parallel fluctuations with age, and the authors think that it probably reaches the cerebrospinal fluid from the brain.

The electric response of the cerebral cortex has been the subject of a good deal of work. One of the most interesting and helpful papers is that of Adrian & Matthews. The subject at present hardly comes within the scope of a review of the biochemical work on the nervous system; but it seems so likely that the gap between the electrical and chemical aspects of brain physiology will be bridged in the near future (in the case of the peripheral nerve, the bridge seems to exist already in the shape of the experiments on veratrinized nerve) that the writer cannot forbear at least a brief reference to some of the points made by Adrian and other workers in the field. The electrical response of the cerebral cortex consists of monophasic waves of higher potential, lower frequency, and longer duration than those in

peripheral nerves. They are abolished within a few seconds by cerebral anaemia (Simpson & Derbyshire). The very long, slow waves which were first identified are due, Adrian & Matthews show, to summation of a number of smaller and more rapid waves, occurring asynchronously in the cortex. This finding serves to diminish, but not to abolish, the difference between the cerebral electric response and that of a nerve.

Injury to the cortex seems to start numerous neurons into synchronous activity, giving waves of a maximum frequency of 60 to 100 per second over an area of 5 mm. or more in diameter. In deep anaesthesia, the waves tend to be slower and more regular than in light anaesthesia, in which latter condition a sensory stimulus may cause irregular and rapid waves. Convulsant drugs, such as thujone (see also Fischer & Löwenbach), may give rise to pulsations spreading widely over the cortex, even when anaesthesia is so deep as to prevent completely any motor response. The great differences between the metabolism of brain and nerve, and the wide differences between the pharmacology of the two tissues, had led to the expectation that electrical investigations would reflect similar differences, differences indeed foreshadowed in Sherrington's theories of excitation and inhibition, and in the conceptions of the Pavlov school. This expectation has been partially realized, though less fully perhaps than might at first have been thought. Adrian, however, points out that the electrical changes observed probably correspond to "spike" potentials and that any "after-potentials" which may exist are not revealed by present methods. It is with the "after-potential" rather than with the "spike" of the electric response of peripheral nerve that recent work teaches us to associate the chemical changes.

Perhaps the most interesting of the recent developments of the chemistry of the central nervous system is that which has established the theory of "humoral transmission" at the nerve endings of the autonomic system, at the synapses of the peripheral ganglia, and at the motor-nerve endings in voluntary muscle. The underlying ideas were clearly in the minds of the pioneers, T. R. Elliott and W. E. Dixon. The crucial experiment was performed on the heart by Löwe; Dale and his collaborators have extended the conception, and have supported it by experimental evidence, until it has become a physiological principle of the first magnitude. A whole review could be devoted to the subject, and it would be useless to try to deal with it in the few paragraphs available here. Fortunately, the omission is immaterial,



in view of the admirable Linacre lecture delivered by Dale. Every worker in the field must be asking himself whether the brain itself is dependent on a humoral mechanism; if so, does that mechanism involve acetylcholine? The question awaits an answer.

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## CHEMICAL EMBRYOLOGY\*

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### THE UNDEVELOPED EGG AS A PHYSICO-CHEMICAL SYSTEM

During the past two years much further attention has been paid to the nature of the phosphoprotein of yolk, vitellin. The combination of phosphoric acid with serine is now generally accepted. Lipmann & Levene isolated phosphoserine from vitellinic acid and Levene & Schormuller found that this compound is not linked in vitellin, as it is in casein, to a dicarboxylic amino acid. Lipmann (2), from a study of the velocity constant of the (monomolecular) splitting of the phosphorus from vitellin, concludes that phosphoserine is the only phosphorus-containing group in the phosphoproteins. He has also, in addition to Posternak & Posternak, worked on the polypeptides (ichthulic acid, ichthiotyrosin) containing phosphorus and some carbohydrate, obtained from fish vitellin (ichthulin). The action of trypsin and pepsin on vitellin was studied by Blackwood & Wishart, who came to the conclusion (in contradiction, perhaps, with Lipmann) that the vitellin molecule must contain at least two widely separated or dissimilar phosphoric complexes, one being highly resistant to enzymic attack. The amino acid distribution of livetin was studied by Jukes, and Fauré-Fremiet investigated the interesting rectangular doubly-refracting platelets of elasmobranch yolk, which consist largely of lecitho-ichthulin, the thuichthin (fish livetin) being in the continuous phase. According to Rae, the glycerophosphoric acid of avian egg-lecithin is entirely in the  $\beta$ -form, in contradistinction to that of brain and liver.

As regards the proteins of egg-white, McNally has found that the thick portion contains a much higher amount of ovomucin than the thin (5 to 9 times). Block found that the lysine content decreased in the order conalbumin/ovalbumin/ovoglobulin, and wishes to apply the orosin concept to the egg-white proteins, suggesting that they are artificial split-products of one entity. An important paper by Sørensen gives new information on the carbohydrate content of these proteins, which ranges from 1.7 per cent in the case of ovalbumin to

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14.9 per cent in the case of ovomucin. This may be compared with similar figures given by Jukes for the yolk-proteins. That the glucosamine in these complex compounds is usually acetylated appears from the paper of Fürth, Herrmann & Scholl. Further work on the occurrence of the remarkable polysaccharide, galactogen, in gastropod eggs has been done by May and May & Kordovich.

The well-known antitrypsin of hen's egg-white is, according to Balls & Swenson, a product of protein hydrolysis found in the thin, not the thick, portion, which acts by displacing kinase from trypsin.

The vexed question of the osmotic relationships in the hen's egg receives even more attention than formerly. As reported in the last of these résumés, Howard obtained, as against previous workers, identical values for the freezing-point depression of yolk and white. This finding has since received a critical re-examination by Smith, who by various new experiments was justified in affirming again the reality of the osmotic pressure difference, and in his work on the sources of error involved could imitate Howard's result at will. Simultaneously Johlin, using the same method as Howard, drew conclusions in agreement with Smith, as also did Weinstein and Hale & Hardy, who took special precautions to avoid injuring the yolk-structure. These latter authors suggested that there might be a gradient of osmotic pressure from centre to circumference of yolk, and this was subsequently shown to be the case by Baldes (e.g., yolk from interior, isotonic with 0.971 per cent NaCl; yolk from vicinity of vitelline membrane, isotonic with 0.844 per cent NaCl; white, isotonic with 0.756 per cent NaCl). Diffusion in the yolk is extremely slow. Mixtures of yolk and white react together so that the osmotic pressure of the mixture is less than calculated. In the meantime, Straub withdrew his original suggestion of thermodynamic work at the membrane. Its permeability to water and various carbohydrates has been studied by Orrù. Salts affect yolk viscosity; this action has been investigated by Zawadzki.

The osmotic situation in invertebrate eggs is considered by Leitch and by Bialascewicz. Leitch determined the non-solvent volume by equilibrium-swelling experiments, and compared it with that deducible from chemical analysis. The discrepancy may be connected with the difficulty of correcting for interstitial water in analyses of masses of minute bodies. There was a correlation between high permeability to water and high protein/fat ratio.

In another communication, Howard attempts to show that during the period of major morphological differentiation, embryos of amniotes are surrounded by fluids very hypotonic to the adult body-fluids.

Turning to the pigments, we find that lipochromes of yolk have been under investigation. It seems that astacin may occur in the eggs of the crab (Kuhn *et al.*), fishes (Emmerie *et al.*; Euler *et al.*), and even in fowls (Brockmann & Völker). The last-named authors relate that only xanthophylls with two OH groups, such as lutein and zeaxanthin, are deposited in avian yolk. If the lipochromes have acquired importance with regard to vitamin A, the flavins have acquired it with regard to the vitamin-B complex. Ovochrome, the yellowish-green flavin of egg-white, has been studied by Kuhn, György & Wagner-Jauregg and by Ellinger & Koschara. Further details will be found in the papers of Stern and his collaborators. The flavins are believed to be connected with the alloxazin series. Their oxidation-reduction is reversible, and Adler & Euler have shown that ovoflavin increases the respiratory rate of bacterial cells by 75 per cent.

In connection with haemoglobin synthesis it is important to know in what form the iron of the yolk exists. Tompsett, using thiolactic acid as a reagent for determination of iron, reports, in confirmation of Hill and against McFarlane (cf. *Ann. Rev. Biochem.*, 2, 338, 1933), that all the yolk iron is in inorganic form, strongly adsorbed on the proteins. Ferric iron added to yolk is at once similarly adsorbed, but can be quantitatively recovered. According to Sherman, Elvejhem & Hart 100 per cent of yolk iron is available for mammalian haemoglobin synthesis, but additional copper is in this case required.

Among the other papers on inorganic materials, those of Straub & Donk and of Busnita & Gavrilescu are valuable. The former authors give an analysis of the ash of egg-white by modern methods; the latter studied the composition of eggs of fresh-water and marine fishes (related species), finding that the former have more ash than the latter (e.g., *Alosa*, in percentage dry weight, fresh-water 6.5, sea-water 5.74). This well exemplifies the greater dependence of marine embryos on the environment [cf. Needham (1), p. 1613 ff.].

A valuable contribution to the chemistry of morphological polarity in the egg is made by Spek in a series of papers where it is shown that considerable pH differences exist within the fertilised egg, and between cells of various presumptive fates at later stages up to and during gastrulation.

## GROWTH AND DIFFERENTIATION

Perhaps the most interesting work in this section is that of Tyler, who measured the oxygen consumption of normal developing sea-urchin embryos side by side with that of "half-embryos," i.e., embryos developing from blastomeres separated in the 2-cell stage. The requisite mass dissociation was brought about by shaking and using calcium-free sea-water. If, it was argued, metabolic work must be done in the production of morphological form, this comparison should reveal it, since exactly twice as much differentiation will be present at the end in the twin case as in the other, per unit mass. Measurement showed that the rate of oxygen consumption was the same within the limits of error for embryos developing from whole eggs and those developing from isolated blastomeres. But as the latter have a delay of 40 per cent in speed of development, the actual amount of oxygen used per embryo in attaining a given differentiation stage will be relatively higher for the half-embryos than for the normal ones. Results on giant embryos made by fusion of normal eggs will be awaited with interest. Another echinoderm study is that of Foerster & Örström, who find that certain doses of potassium cyanide, besides lowering respiratory rate, cause a shrinkage which by vital staining experiments turns out always to be at the ventral pole. Evidence was adduced for believing in the determination of the pole much earlier than generally thought. Specific effects on the amphibian embryo have been discovered by Lehmann: trichlorbutylalcohol inhibits the formation of lens from ectoderm (2), and lithium chloride can completely suppress the formation of the notochord (1).

The importance of the micro-structure of the medium of embryonic cells appears from a review by Weiss (1) on functional differentiation and the rôle of ground substances in development. The orienting effects of micellar aggregates *in vitro* are described in an experimental paper from the same author (2). Chemical and electrical stimuli seem to be much less important in the growth of cell-fibres; and the crystal physics of the protein chain may turn out to be as important in embryology as it has been, for instance, in muscle chemistry.

Light is thrown on one aspect of organogenesis by the discovery of Hueper & Russell and Parker that isolated blood cells are capable of constructing under certain conditions highly organised tubules, the walls of which consist of leucocytes. Here there is some contradiction with the work of Tur, who believes that in the chick the develop-

ment of blood cells and vessels is quite independent. Parker has also studied the growth rate and other qualities of explants of various embryonic tissues, and finds that their growth-rate *in vitro* does not necessarily reflect the age of the donor, but rather the tissue taken.

The relation of the nucleus to development is fundamental. Moore and Francesco show independently by crosses that the cleavage rate of eggs depends on the egg and not on the sperm, probably on the egg cytoplasm. Hämmerling, by working with an enormous and highly differentiated algal cell, *Acetabularia*, could observe the influence of the nucleus on regeneration and morphogenesis.

A particularly important contribution to our knowledge of embryonic growth has been made by Teissier, who finds that when as many entities as possible, both morphological and chemical, are plotted heterogonically against totality, for the rat or for the chick embryo, the changes of slope of all the curves tend to come at the same places. In avian development, these places occur at 1.7 and at 12.0 gm. body weight, i.e., at the ninth and fifteenth days of incubation. On this evidence, three successive plans, manifested by chemical constitution as well as by morphological form, are followed during development.

Work on the mathematics of growth has progressed considerably, but is still in a very individualistic stage. The transition from chemistry is made in the reviews of Rahn and Hueper, who discuss the relation of reduction potential and sulphydryl compounds to growth, the bios factor, vitamins, and auxin. Backman devotes a long review to the mathematical aspect, followed by polemical notes. Mathematics are applied to the growth of explanted embryonic cells in the papers of Mayer and Ephrussi & Teissier, and Ephrussi's book (2). Mathematical accounts of embryonic growth which pay especial regard to the metabolic aspect are those of Bertalanffy and Wetzal. The former explores once more the possibilities of the mass action law, making the hypothesis that anabolism is proportional to the surface and catabolism to the mass. Metabolic rates calculated on this basis from theory are shown to agree with observed values. Wetzal, on the other hand, introduces essentially new and very interesting concepts into his scheme, such as inductance, permittance, and resistance, but he also is largely concerned to bring metabolism into the same quantitative field as growth. Both these systems, fresh and valuable as they are, avoid the question of the mathematisation of differentiation.

That regeneration involves processes of determination and induction analogous to those of normal development becomes ever more



certain, as is shown in the lucid review of Woerdemann. A group of workers, mostly in Russia, have attempted to study the concomitant chemical factors. Thus Bromley & Orechovich find differences between the autolytic processes and tissue cathepsins of normal and regenerating tissues. Orechovich & Bromley show that the blastema can histolyse skin flaps grafted on top of it. Orechovich reports a sulphhydryl content of the blastema much above that of normal tissues. The effects of dehydrating (Nasonov) and X-raying (Butler) the blastema are described. Lastly, Tokin and Cohen review our knowledge of the origin of the cells of the regeneration blastema.

A beautiful study of the development of function is that of Chambers & Kempton, who isolated embryonic mesonephric tubules in tissue culture and found that after the ends had closed the hollow structures so formed could accumulate phenol red and water in their lumina against a considerable concentration gradient.

#### ORGANISER PHENOMENA

Since the discovery in 1924 by H. Spemann & H. Mangold that the dorsal lip of the amphibian blastopore would, if transplanted so as to be in contact with competent ectoderm, induce in the host the formation of a secondary embryo, there has been much speculation about the nature of this organiser action. At the time of the publication of the last of these reports, it was known that the integrity of the cells was not necessary, since the organisation centre would induce after the crushing of the cell structure (Spemann, 1931; cf. Krämer), and that normal respiration was unnecessary, since induction by deeply narcotised organisers was possible (Marx). It was next shown that the organiser would induce after complete coagulation by boiling (Bautzmann *et al.* for the newt, Waddington (1) for the chick). This strongly indicated the presence and activity of one or more thermostable chemical substances. The demonstration that the organiser could be obtained active in cell-free extract was then given simultaneously by Waddington, Needham & Needham, by Holtfreter (2), and by Spemann, Fischer & Wehmeier. The first-mentioned authors further showed that the organiser was soluble in ether and petrol-ether.

Holtfreter (1) then reported the remarkable fact that not only is the organiser not destroyed by boiling, but that those parts of the embryo which normally show no inducing activity, such as the ventral ectoderm, acquire it on being boiled. The organiser substance may thus be liberated from some masking complex. Holtfreter (2) also

was able to show that all tissues of adults throughout the animal kingdom contain the organiser in active condition. No vegetable or inorganic material of any kind can act as organiser. The organiser is destroyed by ashing. This wide distribution of the active substance explains the striking lack of species-specificity of organisers, noted by embryologists earlier [e.g., chick blastoderm induces a secondary embryo in rabbit, Waddington (2); and in newt, Hatt]. Regeneration blastema (Umanski) and tumour-tissue [Woerdemann (3)] also contain it. Only in the non-inducing parts of the normal egg is the organiser masked.

Next came an announcement by Fischer & Wehmeier that induction could be brought about by glycogen, conflicting with the ether-solubility of the active substance. Waddington, Needham, Nowinski, Needham & Lemberg, however, were able to prepare very active fractions by extracting glycogen obtained by the classical potash method, with ether. It is therefore certain that an ether-soluble material is thrown down with the glycogen in the alcohol precipitation. Waddington, Needham, Nowinski & Lemberg were further able to show the existence of the organiser in the unsaponifiable fraction of mammalian liver, from which it appears to be precipitated by digitonin. Strong evidence that the naturally occurring organiser is contained in the unsaponifiable fraction is provided by the work of Waddington & Needham, who tested some of the synthetic hydrocarbons prepared by Cook *et al.* in connection with carcinogenesis and the oestrus cycle. While oestrin, calciferol, etc., were inactive, 1,9-dimethylphenanthrene, and 9,10-dihydroxy-9,10-di-*n*-butyl-9,10-dihydro-1,2,5,6-dibenzanthracene were able to induce a secondary neural axis in the amphibian embryo. The oestrogenic hydrocarbons appear to be much more active as organisers than the carcinogenic ones. Barth has recently found the cephalin fraction of mammalian brain to be active, but many other substances are present in this fraction, when unpurified.

The action of organisers is closely connected with the facts which lead to the concept of morphogenetic fields, as the books recently written by Huxley & de Beer and by May show. Embryonic induction is divided by Needham, Waddington & Needham into "evocation," the determination that an embryonic axis shall be developed, and "individuation," the determination of the regional, e.g., antero-posterior, character of that axis. It is doubtful whether individuation can be performed by dead organisers, organiser-extracts, or single chemical

substances; the substance studied in the foregoing investigations may therefore be called the "evocator." Waddington (3) has given, recently, a theoretical discussion of the field-concept. Its further elucidation along physico-chemical lines is perhaps the outstanding problem of embryology (cf. Balinsky on limb fields).

The extremely wide range covered by organiser phenomena is demonstrated anew by the important work of Seidel and Schnetter on insect embryos and of Oppenheimer on those of fishes.

#### RESPIRATION AND GLYCOLYSIS

Contributions to this field have been both numerous and important. We may begin with the insect egg, hitherto relatively little investigated.

The eggs of Orthoptera, e.g., grasshoppers, may undergo a long period of quiescence during their development, growth and differentiation ceasing, and metabolism being very reduced; this is the diapause. Bodine (1) and Slifer have examined the effects of hypertonic solutions on respiratory rate in these eggs, and the relation between oxygen tension and oxygen uptake has been studied by Bodine (3), but most progress has been made in the elucidation of the diapause mechanism. Bodine (2) first found that the respiration of developing eggs is cyanide-sensitive, whereas that of diapause eggs is not. The parallel with the fertilised and unfertilised echinoderm egg is striking. Bodine & Boell (1) went on to show that the respiration of developing eggs is inhibited by CO/O<sub>2</sub> mixtures proportionally to the carbon monoxide concentration, but that the diapause eggs are specifically insensitive to carbon monoxide. Bodine & Wolkin found no difference in the iron content of developing and diapause eggs; it increased always in proportion to the mass of the embryonic body. The degree of saturation of the *Atmungsferment* must therefore account for the facts. Finally, Bodine & Boell (2) showed that the respiration during diapause is qualitatively and quantitatively identical with the cyanide-insensitive respiration during development. The factors producing diapause must therefore include some agency which suppresses the cyanide-sensitive fraction of normal respiration. In addition to all this work, Burkholder has followed the respiration of single orthopteran eggs throughout development, and Evans has shown that there is no decrease of sulphhydryl at the time of diapause.

On the classical material, the eggs of echinoderms and worms, still more has been done. According to Whitaker (1), in continuation of

studies previously referred to, the oxygen uptake of *Chaetopterus* is lowered by 50 per cent on fertilisation and increased by 400 per cent in *Arbacia* (100 per cent in *Psammechinus*, Borei). Whitaker (2) gives a review of all work on this subject, and concludes that the values, very variable among the species before fertilisation, tend to reach a common level after it, by rising or falling. Gerard & Rubinstein dispute, however, the absolute values of Whitaker on technical grounds, suggesting that they should all be 80 per cent higher. Rubinstein & Gerard show that whether or not a rise occurs on fertilisation depends on the temperature. The  $Q_{10}$  is 4.1 before and 1.8 after fertilisation in *Arbacia*. They suggest that the former "physical" value indicates accessibility of the *Atmungsferment* to be the limiting factor, while the latter "chemical" one indicates speed of reaction. The R.Q. during fertilisation has been given as 0.92 (van Herk), 0.9 (Borei) and from the 2-blastomere stage to the hatching blastula as 0.8 [Ephrussi (1)]. The last-named author found that the total amount of oxygen consumed and the R.Q. at different temperatures are the same. That reversible dyes increase the egg respiration has been shown by Barron & Hamburger; Ellis; Brooks; and van Herk. The increase is not inhibitable by cyanide, and is larger in the unfertilised eggs than in the fertilised ones.

Ellis also reports that echinoderm and cephalopod cleavage rates are not decreased by iodoacetate or fluoride, nor accelerated by cystine or glutathione. He calculates that the energy required for increase of surface is exceedingly small in comparison with the turnover of respiration. The curious question of the fixed acid liberated after fertilisation has been pursued by Borei and by Runnström, who find it unaffected by anaërobiosis and producible by hyper- and hypotonic solutions as well as fertilisation. It is not accounted for by phosphate or lactic acid metabolism. Anaërobiosis, however, reduces the permeability constant of the eggs by 24 per cent (Kekwick & Harvey) but has no effect on the equilibrium volume. The effect of lithium has been studied by Lindahl. Tchakhotine observed that in eggs treated with bromoacetate there is a suppression of endoderm and stimulated proliferation of mesenchyme, side by side with the reduction of glycolysis.

The aerobic eggs and the anaërobic adults of the tapeworm *Diphyllobothrium* have been found by Friedheim & Baer to have a respiration wholly uninhibitable by carbon monoxide. Yet potassium cyanide completely inhibits the egg respiration, only partly that of the adults.

The oxygen uptake and R.Q. have been determined on eggs of two fishes: for *Fundulus*, Amberson & Armstrong find an R.Q. falling from above 0.9 on the first day to somewhat above 0.7 for most of the period of development; for *Salmo*, Schlenk finds an R.Q. of 0.65 to 0.72 for the latter part. These figures agree with the hypothesis of a succession of sources of energy during development [Needham (1), pp. 986 ff.]. Manery, Warbritton & Irving studied the genesis of an alkali reserve in *Fundulus* eggs and found the bicarbonate content to increase most rapidly during periods of high respiratory activity. The total increase was 3 to 18 cc. of  $\text{CO}_2$  per 100 gm. of eggs. Irving & Manery showed that a chloride loss exists side by side with this bicarbonate gain. They give a penetrating discussion of ionic equilibria found during embryonic development. Compare with this Brinkman's discovery of carbonic anhydrase in eggs.

Brachet (2) has carried out very extensive work on the frog embryo. Segmentation and early gastrulation can take place anaerobically; neurulation is impossible without oxygen. By potassium cyanide the respiration can be reduced by 90 per cent with segmentation unaffected, but phenylurethane stops it immediately. Iodoacetate has relatively little effect till late gastrulation. The R.Q. is unity before fertilisation, drops during segmentation to 0.7, and rises again to unity for the rest of development. No lactic acid accumulates aerobically, and very little anaerobically. Instead of glycolysis, an oxidising reserve like that of nerve seems to exist, as Brachet proves by demonstrating an oxygen debt, during the paying off of which the R.Q. is 0.3. Cytolysis of the gastrulae increases the oxygen uptake fivefold, but has no effect on that of the embryos at hatching. Since para-phenylenediamine raises the respiration of the cytolysed material no further, the saturation of the *Atmungsferment* must be in question (cf. the unfertilised sea-urchin egg and the insect diapause). The fertilised frog egg contains no cytochrome and very little glutathione. On fertilisation there is no change in the respiratory rate. Some of these facts are confirmed and extended by Lennerstrand and Demuth in independent investigations.

Brachet (3) also finds during amphibian mitosis irregularities in the oxygen uptake which may be due to rhythmic processes. He also reports (4) the very interesting fact that the organiser centre has a higher respiratory rate than any other part of the embryo. If pieces of dorsal blastopore lip and ventral ectoderm are removed from a series of embryos, those which have lost the former show less  $\text{Q}_{\text{O}_2}$ .

than those which have lost the latter. The organiser centre, too, directly measured, shows a higher carbon dioxide output. This discovery may throw light on the means whereby the organiser arises in the dorsal lip, and is the first piece of evidence in favour of the respiratory gradients of Child in embryonic material.

Progress has also been made with the chick embryo; thus Fukahori observes a falling R.Q. in the intact egg [agreement with earlier work, cf. Needham (2)]. Needham (2) has studied the R.Q. of the membranes; that of the allantois falls slowly from 0.95 to 0.82; that of the yolk-sac falls sharply from 0.9 to 0.6 or below from the seventh day onward. It could not be proved that this was due to a conversion of fat to carbohydrate, though the histochemical work of Jacobson makes this very likely. The absorption rate and transit rate of the yolk-sac have been calculated. A high R.Q. is associated with the formation of the yolk-sac rather than with its function. Dickens & Greville (1) find that the respiration of embryo tissues falls very little when they are deprived of glucose; they contain reserves of whose nature we are ignorant. They readily oxidise fructose.

A series of papers by Lipmann (1), Laser, and Havard & Kendal consider the facultative anaërobiosis of embryo cells in tissue culture and its relation to the rH of the medium. Growth is certainly possible in absence of oxygen. Enzmann & Pincus find that the latent period between decapitation of a mammal and the disappearance of all spinal reflexes decreases regularly with age from birth. Is this the reflection of that capacity for anaerobic life shown in Laser's work?

Finally, the behavior of a platinum electrode immersed in a suspension of echinoderm eggs, simultaneously cleaving, is described by Reiss.

### METABOLISM

*General surveys.*—For molluscan eggs we have work by Stolfi and Konopacki (histochemical) on cephalopods, and by Kumon and Baldwin on gastropods. Stolfi reports increases in total weight, water, and ash, of the whole egg during the development of the squid, *Loligo*, with loss of both protein and fat. Kumon's work was along similar lines, but Baldwin observed that the R.Q. of *Limnaea* eggs is maintained at unity throughout their development, side by side with a synthesis of fat sufficient to mask any fall of the quotient. For the silkworm we have a lengthy report by Akao, but his figures seem to

be urgently in need of confirmation, since an actual decrease of uric acid is reported. The whole subject of the nitrogen metabolism of the insect egg needs, in fact, a convincing study, which is not provided by the papers of Manunta or Tanda.

Salmon development has been studied by Allen, who finds no water uptake before hatching, while Konopacki & Erecinski have applied histochemical methods to a teleost with paternal pouch incubation, *Syngnathus*. For amphibia there is the work of Fujiwara *et al.* on the Japanese salamander; it appears that during development 32 per cent of the fat, 26 per cent of the nitrogen, and 44 per cent of the glycogen are lost. Cholesterol decreases, and its esters increase. Data for ash, phosphorus, etc., are also given.

There are new data, too, for the composition of the human embryo (Iob & Swanson; Givens & Macy) but it will be long before our knowledge in this field is statistically satisfactory.

It is of interest that according to Holmes,  $\gamma$ -radiation from radium reduces the carbohydrate catabolism of embryonic rat kidney in tissue culture by 50 per cent without affecting its protein breakdown at all.

*Nitrogen*.—Dickens & Greville (2), studying the early chick embryo in the absence of sugar, find that aerobic ammonia formation can be large [agreement with Needham's inhibition experiments (2)]. Although *in ovo* the amounts of urea produced are considerable in relation to the total nitrogen excretion, it has been found by Needham, Brachet & Brown that this urea is solely derived from arginine, not from ammonia by the ornithine cycle, nor from uric acid by Stransky's uricase. It accumulates steadily and contributes nothing to later uric acid formation. The enzymes of the embryo are described by Borger & Peters; in extracts, dipeptidase and aminopolypeptidase appear, but no proteinase. Borger & Zenker show that the growth-promoting activity of embryo extract goes parallel with its dipeptidase content. The proteinase of yolk-sac extract is twelve times as active as that of embryo extract.

Other interesting work on the enzymes of the embryo is that of Linderstrøm-Lang, who measured the peptidase activity of single worm and echinoderm eggs, and of Philipson, who found it to be equally distributed between the fragments of centrifuged echinoderm eggs. Truszkowski & Czuperski report that Stransky's uricase appears only relatively late in amphibian development, at the time of retraction of the external gills (fifteenth day).

In the hope of increasing the amount of creatinine formed by the



chick embryo, Takahashi injected a variety of substances, especially amino acids, into the egg; arginine and creatine alone were effective. Extra creatinine injected by Ono was destroyed.

Contrary to Brachet's finding (1) of an increase of thymonucleic acid and a decrease of phytonucleic acid during development of the echinoderm egg (see *Ann. Rev. Biochem.*, 2, 343, 1933) Schmidt has obtained a positive Feulgen test on a purine-containing fraction of these eggs after fertilisation. But this is probably not significant, as Marza & Marza can demonstrate a weakly positive Feulgen test on hen's egg-yolk, which certainly contains no thymonucleic acid. The teleost egg has been studied by Ghinst, who reports a rise of both phyto- and thymonucleic acid during development. This is similar to what takes place in the chick embryo, and negatives the correlation between uricotelic catabolism and nucleic synthesis [Needham (1), p. 1159].

*Carbohydrate.*—The largest piece of work on this subject, other than those already referred to in connection with glycolysis, is that of Donhoffer, who has examined afresh the changes in the various carbohydrate fractions during the development of the chick. Earlier workers are in the main confirmed. Free sugar is at a minimum on the tenth day; the glycogen originates mainly from a fraction insoluble in alcohol or water (protein-combined); total sugar diminishes till the ninth day and remains constant afterwards; during this time it is probably being formed from something else, probably fat (see above, p. 459), thus masking a disappearance by combustion.

As one of the substances most readily observed histochemically, it should be of interest to study the distribution of glycogen in regions of importance for experimental morphology. Woerdemann (2) accordingly found that the cells of the roof of the archenteron in the frog embryo are free from glycogen which has disappeared from them during their passage through the lips of the blastopore. Acquisition of induction-power is associated, then, with loss of glycogen, and so is invagination, for Raven found that any dorsal lip, invaginating in a host, loses glycogen as it does so. According to Woerdemann (2), glycogen also disappears from the eye-cup during the induction of the lens. These important studies are being extended to the chick by Jacobson and to the mammal by Aloisi (1).

Metabolism of glycogen in the cephalopod embryo is considered by Chaigne. The glycogen of embryonic tissues, according to Aloisi (2), is much more resistant to autolysis than that of the adult; and

in the placenta, according to Davy & Huggett, it yields glucose, not lactic acid on autolysis (resemblance to liver, not muscle). Harris, studying the glycogen in hypertrophic cartilage in mammals, points to a relation between this carbohydrate store and the hexosephosphoric esters which are needed at immediately adjacent points as substrates for Robison's phosphatase in calcification. The more senescent the cartilage cell, the more glycogen it contains. It is remarkable, in this connection, that Robison & Rosenheim find iodoacetate and fluoride to have a powerful depressant effect on part of the calcification mechanism.

Fructose, hitherto known only in mammalian allantoic liquid, has been shown to occur also in that of the chick by Yamada, and Donhoffer brings evidence for its existence among the sugars of the egg in the early stages of incubation. Its close connection with embryos remains mysterious.

*Fats and lipoids.*—Whereas from the older studies in the literature it has appeared that the triglycerides and the phosphatides followed during the chick's development a different course of breakdown with time, Jost & Sorg now clearly show the existence of a far-reaching parallelism. Though both are reduced by 60 per cent before hatching, the ratio between them remains the same throughout. Iodine values of triglyceride and phosphatide fatty acids both within and without the embryo at different stages were determined; in the whole egg the iodine number of the neutral fats remains at about 75, that of the phosphatides falls from 70 to 50, approximately; at the end of development the fat of embryo and remaining yolk has the same value, but the iodine number of the embryo-phosphatide tends toward 60 and that of the yolk tends toward 40. The interpretation of all these facts is still uncertain, but the authors support the view that phosphatide is a necessary stage in the transport and oxidation of the fat in the egg. Some further light on this, but not much, may be obtainable from histochemical methods, in which connection the work of Konopacka on the chick embryo is to be recorded.

That the fat in the bodies of developing mammalian embryos may be affected according to the composition of the diet and hence of the fat depots of the mother, is again affirmed by various authors (Chaikoff & Robinson; Sinclair). This proves the correctness of the view [Needham (1), p. 1524] that there is a slow but definite and continuous passage of fat through the placenta, rather than a new formation of fat from other substances by the foetal tissues.

*Water and ash.*—Numerous studies of the inorganic constituents of embryos have appeared, but where the same metal has been investigated by more than one worker, the agreement between the results is not always exhilarating. They may be summarised thus: chlorine in the chick embryo is negatively heterogonic<sup>1</sup> (Yamada) [ $k = 0.78$ ; cf. the views of Irving referred to above (p. 458)]. Iron in the chick embryo also increases more slowly than the dry weight ( $k = 0.87$ , McFarlane & Milne; Szejnman-Rosenberg), but in its membranes, while they last, increases more rapidly ( $k = 1.13$ ). Here the figures of Katsunuma & Nakamura are quite aberrant. Calcium in the turkey embryo (Insko & Lyons) gives  $k = 1.15$ , agreeing exactly with other embryos, and copper in the chick embryo "grows" at nearly the same rate as the iron ( $k = 0.85$ ; McFarlane & Milne). In the pig embryo it has been studied by Wilkerson. The subject as a whole is considered by Ranzi, who seeks some explanation of the universal fall of ash content during development, and by Ramage *et al.*, who apply spectrographic analysis to embryos, revealing the presence and quantity of metals both common and rare.

Under the head of water metabolism, mention of a lethal gene ("sticky") discovered by Byerly & Jull cannot be omitted. Chicks with this gene fail to absorb the water of the amniotic and allantoic liquids before hatching.

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<sup>1</sup> For explanation of this terminology see *Biol. Rev.*, **9**, 79 (1934).

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THE BIOCHEMICAL LABORATORY  
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## THE BIOCHEMISTRY OF MALIGNANT DISEASE\*

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Many advances have been made recently in the knowledge of tumour-producing chemical substances and also of the production of tumours by viruses. It is impossible to know as yet whether it will eventually prove that these two methods of attacking the cancer problem can be combined to bring us to the solution, but there is undoubtedly a possibility that this will be so. The work of McIntosh (1), who has produced tumours in fowls by painting them with carcinogenic substances, and has then found that some of the tumours are filterable, that is to say that they contain what is commonly supposed to be a cancer-producing virus, is very suggestive of such a possibility.

### CHEMICAL SUBSTANCES PRODUCING CANCER

The recent work on carcinogenic substances has been summarised by Boyland in the *Annual Review of Biochemistry* last year, and also by Dodds (2) who gives a full account of the relationship between the bile acids and sterols, such hormones as resemble these chemically, and the carcinogenic substances.

Cook & Haslewood (3) [see also Cook (4)] have described some very important work showing that an exceptionally potent carcinogenic substance, methylcholanthrene, can be made from the bile acid deoxycholic acid. Dehydronorcholene is an intermediate product and the essential steps in the transformation are cyclisation of the side chain and dehydrogenation of the ring system.

Twort (5) has found that any process tending to saturate or hydrogenate lubricating oil makes it less carcinogenic; this agrees with the experimental work of Cook and others, who find that an unsaturated ring system is necessary for carcinogenic activity in the substances they have studied.

Pourbaix (6) has obtained rather irregular results suggesting that benzpyrene may cause a lowering of respiration and increase of glycolysis in some surviving tissues, particularly brain tissue.

Campbell has produced lung and skin tumours in a considerable percentage of mice exposed to dust from tarred roads (7). He states

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that the "dusting" received by these mice and the length of exposure to it is much greater than any human being would be at all likely to receive, so that it is difficult to gauge the importance of road tarring as a factor causing tumours in humans.

#### BIOCHEMISTRY OF THE FILTERABLE TUMOUR-PRODUCING AGENTS

A considerable number of filterable fowl and duck tumours are now known; the first to be described was that discovered by Rous about twenty years ago. The tumour-producing agent can be extracted from these tumours in saline solution, and can be passed through a Berkefeld candle or a paper-pulp filter. It is now commonly believed that the infective agent is a virus, but Gye & Purdy (8) have suggested that the specificity of the agent, which enables it to reproduce exactly the same type of tumour in the new host as that from which it is taken, is due to some chemical factor which accompanies the virus. The biochemical approach to the problem has been directed chiefly toward purification of the agents, and toward determining what chemical substances or enzymes will cause the destruction of the agents.

Murphy (9) has found that practically the whole tumour-producing power of a filtered tumour extract can be removed by precipitating it at a pH between 4.2 and 4.8; the redissolved precipitate will produce tumours. At pH's below about 4.0 the agent is destroyed. The redissolved precipitate contains protein, which Murphy has been able to remove by adsorption with alumina-*c* gel. The supernatant fluid from the alumina contains a substance resembling chondroitin sulphuric acid, which can be removed by the addition of gelatine. The supernatant fluid from the alumina gel does not give the chemical tests for proteins, and produces very little anaphylactic sensitising effect in guinea-pigs, but is very active in producing tumours. In Murphy's experiments most of the active substances are left in the supernatant fluid, but other authors have found that a large proportion of the tumour-producing agents can be adsorbed on alumina-*c*- $\gamma$  gel and afterward eluted with alkaline phosphate or very dilute ammonia. By this means purified solutions of the tumour-producing agents can be obtained. Maschmann (10) has used this method and also Lewis (11), who has found that she can obtain extracts which do not give chemical tests for protein but are active in producing tumours.<sup>1</sup> Pirie (13) has

<sup>1</sup> Nakahara & Nakajima have also shown that the Rous agent can be adsorbed on aluminium hydroxide, but the loss is too large to permit use of this method for purification (12).

found that a considerable proportion of the tumour-producing power is removed by adsorption with the  $c$ - $\gamma$  gel and that active tumour-producing solutions can be obtained by elution from the alumina with sodium phosphate at pH 9.2. If too much alumina is used the tumour-producing power cannot be recovered. The amount of nitrogen in an active dose of the eluate is only about  $10^{-3}$  mg. The eluates, like the original filtrates, lose their potency on standing and are preserved by the addition of cyanide.

A summary of the literature on this subject is given by Claude & Murphy (14) in their review. Murphy himself has suggested that the transmissible agent cannot be a virus, as it withstands very severe treatment, but other viruses have been found to be equally resistant [poliomyelitis virus, Clark, Schindler & Roberts (15); bacteriophage, Kreuger & Baldwin (16)].

Murphy & Sturm (17) have shown that an anti-infectious agent is also adsorbed on the alumina gel at pH 7.0, and can be eluted from it with basic sodium phosphate at pH 9.0. Heating at  $52^{\circ}$  for thirty minutes destroys any tumour-producing activity that might be present, leaving only the inhibitory agent. Murphy & Sturm (18, 19) have also shown that the anti-infectious agent is effective in preventing the growth of one transplantable mouse sarcoma, though not that of two other mouse tumours. This suggests the possibility of a relationship between the filterable fowl tumours and the ordinary mammalian tumours.

A discussion of the evidence for the possibility that mammalian cancer is also a virus disease is outside the scope of this review; the possibility [Gye (20)] has long been recognised, although the view has not been generally accepted. Reference must, however, be made to recent work of Rous & Beard (21) with the filterable wart of a cotton-tail rabbit. This tumour behaves in most respects like a true malignant growth when actual wart tissue is injected into various organs of the body or into the blood stream, and causes the death of the animal. The wart-producing filtered extracts, on the other hand, show the complete specificity found with filtered extracts of chicken tumours, and will infect only the skin. Moreover after inoculation into a domestic rabbit the tumour becomes non-filterable and behaves like an ordinary transplantable mammalian cancer. Thus this tumour forms a link between the filterable fowl tumours and the mammalian tumours. The implications for general theories of cancer which are brought out by these results are very clearly discussed by Rous & Beard.

The spontaneous disappearance of tumour-producing activity from filtrates of Rous sarcoma, which is usually complete in 24 hours at 37.5° C., has been studied by Pirie & Holmes (22). Although the activity is lost even in the absence of air, they consider that this destruction is due to oxidation, as it is prevented by the hydrogen-palladium system, and the tumour-producing power is partly restored after loss by cobalt plus cysteine. Sulphite and cysteine have a preservative effect anaerobically, but accelerate destruction aerobically. Methylene blue accelerates the destruction even in the absence of air. The well-known preservative effect of cyanide can be accounted for either as an effect on oxidative catalysts or an effect on proteolytic enzymes.

Many attempts have been made in recent years to discover something of the nature of viruses by attempting to inactivate them with different enzymes. In some of these the results are not clear owing to the fact that commercial trypsin or pancreatin have been used, which are, of course, mixtures of enzymes. This applies to the work of Sugiura (23) who has found that pepsin at pH 3.8 and trypsin at pH 8.2 inactivate Rous-sarcoma filtrates, while the heat-inactivated enzymes do not do so. Soy-bean urease, takadiastase, and castor-bean lipase, tested at their optimum pH, have no effect. Fränkel (24) has found that trypsin does not destroy the infectivity of either Rous-sarcoma cells or filtered extracts. Baker & McIntosh (25) have obtained results which are in agreement with those of Sugiura, as they have found that commercial trypsin at pH 8.0 inactivates the Rous-sarcoma filtrates. Work with other viruses has given equally discrepant results; this work is summarised by Pirie (26). Pirie has used a sample of Merck's pancreatin, which contains active protease, carboxypeptidase, amylase, and lipase. A saline suspension of this pancreatin inactivates Rous- and Fujinami-sarcoma filtrates. When a glycerol extract of the pancreatin is fractionated by adsorption with alumina-*b*, the supernatant fluid, which contains the protease, carboxypeptidase, and amylase, is found to have lost its inactivating power. The eluate from the alumina-*b*, which contains lipase, can still destroy the tumour-producing power of the filtrates. It is shown that the enzyme responsible is not lipase, as fresh pancreatic juice activated by enterokinase, which contains active protease, carboxypeptidase, and lipase, does not destroy the tumour-producing power. By adsorption on casein (unpublished work), Pirie has found it possible to obtain the enzyme in a relatively concentrated and heat-stable form, so that

it is possible to free it from most of the ordinary pancreatic enzymes. This fraction is active against the chicken-tumour viruses and against other viruses as well.

#### ENZYMES IN TUMOURS AND IN THE TISSUES OF TUMOUR-BEARING ANIMALS

Edlbacher, Kraus & Leuthardt (27) have confirmed the earlier finding of Edlbacher and co-workers that rat tumours contain a large amount of arginase. In air, the optimum pH of tumour arginase is not the same as that of normal tissue arginase. This seems to be due to some indirect effect of tumour metabolism and not to any real difference in the enzymes, since increasing the activity of the enzyme by excluding oxygen or adding SH groups also shifts the pH optimum to the normal reaction. The arginase-activity of tumours can be increased 400 to 600 per cent by these methods, whereas that of normal tissues is increased, at most, 50 per cent. McFadyen (28) has also confirmed the statement of Edlbacher & Kutscher that tumour extracts (chicken tumour *I*) contain phosphatase and nucleotidase in amounts which are about equal to those in spleen extracts and much greater than the amounts in muscle extracts. He has also found a rough proportionality between nucleotidase activity and tumour-producing activity in different fractions of the chicken-tumour-*I* extracts. Edlbacher & Kutscher (29) had found that not only did the tumours themselves contain nucleophosphatase but that the muscles of tumour-bearing animals contained amounts of the enzyme considerably in excess of the normal. A similar effect of tumour growth on distant organs of the body has been demonstrated by Maschmann & Helmert (30, 31) who have found that the amount of cathepsin in the liver and spleen of tumour-bearing animals increases during the course of tumour growth. The amount of cathepsin in the tumour itself, on the other hand, remains low, particularly in the necrotic portions, and is rather less fully activated than that in normal tissues. Mouse carcinoma and mouse and hen sarcoma were used.

Bernhard (32) has found that the level of atoxyl-fast lipase (pancreatic lipase is relatively atoxyl-fast) in the serum is considerably above the normal in cancer, and falls after successful treatment. The total lipase of the serum had, however, been shown by Shaw MacKenzie, whose work was confirmed by Watchorn of this laboratory (unpublished results), to be definitely lowered in cases of cancer. Green (33) has found that in rats with implanted tumours the esterase

content of the serum falls during tumour growth, the level being definitely lowered even in the early stages when the rats are perfectly healthy, and very low indeed when the tumours are large. He has not, however, found this in other animals or in human patients. Some of the discrepancies between the results of different workers can probably be explained by the fact that they have not used the same substrates in estimating the lipase. Green used chiefly ethyl butyrate, and found that tributyrin and other esters gave the same results; olive oil has been used by other observers.

Braunstein & Heyfetz (34) have found that the glycolytic power of the blood of rats with implanted carcinomata is normal and Tu-reen (35) has not been able to find any constant alteration in the serum-diastase level in cancer.

It has long been known that cancer tissues in tissue cultures have an abnormally high potency in liquefying the plasma clot upon which cultures are grown. Demuth & Riesen (36) have shown that this is due to the outward diffusion of a heat-stable activator, which activates an enzyme in the plasma. Normal tissues contain this activator, but to a smaller extent, and as fewer normal cells die in culture the amount of activator escaping is probably much less than in cultures of tumour cells. The action of the activator can be imitated by copper salts, but not by anything else investigated.

#### GENERAL CONSIDERATIONS OF TUMOUR METABOLISM

Some investigations have been made of the effect of growth factors on tumour growth. Interest in the effect of the pituitary on tumour growth was aroused by reports, probably ill-founded, of clinical cures. Recent papers on the subject are those of McEuen & Thomson (37) and Engel (38). McEuen & Thomson have removed the pituitary from rats and shown that when the growth of the rat is stopped the growth of the tumour is also slower; this effect is not specific, as the growth rate of the tumour is similarly decreased when the food intake is sufficiently restricted to stop the growth of the animal. Engel has found that injection of the growth factor of the anterior pituitary has a somewhat accelerating and of the gonadotropic hormone a somewhat inhibiting effect on tumour growth.

Fodor has claimed that large doses of vitamin C, either injected or given in the diet, cause a great increase in the growth rate of the Ehrlich tumour (39).

Several authors have considered the possibility that a growth fac-



tor contained in the liver [see Mapson (40)] has an effect on the growth of tumours. Haddow (41) has given a short review on the subject, and quotes Maisin & François (42), who have shown that when animals are fed on liver during tar-painting the death rate from cancer and the metastasis rate are increased. The criticism can be made that their control animals were fed on a deficient diet; the work, however, has been partially confirmed by Watson (43).

It has long been known that some nitrophenols can increase the metabolism of the whole animal, and recently it has been suggested that direct stimulation of oxidative mechanisms in the cells can be produced by them [Magne, Mayer & Plantefol (44)]. Emge, Wulff & Tainter (45) have investigated the effect of injecting dinitro-phenol into rats upon the growth of an implanted fibrosarcoma, but have not obtained any very marked results. Dodds & Greville (46) have shown that the metabolism of tumour slices can be very greatly increased by the addition of  $10^{-8}$  *M* dinitro-*o*-cresol in phosphate-Ringer solution containing glucose, pyruvate, or lactate. Without added substrate there is no effect. They have also demonstrated the remarkable fact that the aerobic glycolysis and the respiration are both much increased by the dinitro-cresol.

Greville has pointed out (unpublished work) that the nitrophenols, unlike the various dyestuffs which increase respiration, are not reversible oxidation-reduction systems, and cannot cause mammalian erythrocytes to respire, but can only increase respiration where it is already present. They are only reduced by dehydrogenase systems in rather special circumstances, and the cause of the increase in metabolism produced by them is therefore obscure. Dickens (unpublished work) has investigated the effect of cresyl-blue thionine and pyocyanine upon the respiration of tumour tissue. These substances he has found to be less toxic than methylene blue, and each produces a very large increase in tumour respiration; the increase is dependent on the presence of glucose, and the R.Q. of the extra respiration is unity. The cancer cell can apparently oxidise carbohydrate completely if it is provided with a suitable oxidation catalyst. The dyes do not cause a simultaneous decrease of aerobic glycolysis. Friedheim has shown (47) that pyocyanine, which increases the respiration of any tissue which normally has an aerobic glycolysis, will sometimes do so. Dickens (48) has shown that if tissues are poisoned with phenylhydrazine the aerobic glycolysis is raised to the level of the anaerobic glycolysis. The respiration of tumour tissue is, however, very little decreased;

this is also true of normal tissues whose respiration is well maintained without the addition of glucose to the medium. Boyland & Mawson (49) have found that they cannot extract the glycolytic system from tumours; tumour tissue cannot glycolyse glycerophosphate plus pyruvate as readily as it can glucose.

Miyao (50) has also carried out respiration experiments with tumour tissues; he has shown that glycine, glutamine, and tyramine cause an increased metabolism when added to rat liver tissue, but not when added to rabbit-sarcoma tissue.

Myer, McTiernan & Salter (51) have investigated a large number of sugars and possible breakdown products of sugars in order to find out which of these can serve as substrates for glycolysis in normal and malignant tissues; they have found no difference between normal and malignant tissues in respect of the substrates utilisable by them. Salter & Robb (52) have also shown that tumour tissue produces ammonia when no substrate is added, and that the substrates which are best used for glycolysis are also those which are most effective in preventing the appearance of ammonia.

Magath, Smailowskaja & Kolomietz (53) find that the addition of monoiodoacetic acid in amounts greater than that required to kill the tumour cells does not increase the amount of phosphate diffusing out of tumour slices. With liver slices the amount is much increased. They consider it unlikely that phosphagen is used as an energy source in tumour tissue.

Bumm (54), in a review dealing with the metabolism of growing tissues, has suggested that not only tumour tissue but all actively growing tissues have an anaërobic type of metabolism, and that the presence of high oxygen pressures is unfavourable to growth. He mentions the fact that the foetus *in utero* grows under conditions of low oxygen tension, and that high oxygen tensions prevent the growth of tissue cultures. He considers that glycolysis rather than respiration is the metabolic process particularly essential for growth. Cancer tissue, of course, differs from most normal tissues in that its aerobic glycolysis is not altogether inhibited by oxygen. He quotes the work of Laser (55) who has recently shown that normal tissues can live, or even grow, in culture without oxygen.

As regards the chemical substances occurring in tumour tissue Bierich & Lang (56) have confirmed Bierich's earlier result and have shown that the cholesterol-ester content of tumours is very high, so that, in spite of a fairly high phospholipoid content, the ratio of

cholesterol to phospholipoid is unusually large. Morávek (57) has carried out analyses of the inorganic substances in tumours.

Roe & Dyer (58, 59) and Dyer & Roe have made very exhaustive determinations of the blood chemistry of hens bearing the Rous sarcoma, and find no change of diagnostic significance. In agreement with them Dickinson & Havard (60), using the glass electrode, find that the reaction of the blood is not altered in human cancer patients. They point out, as a possible source of error in the work of other authors, that even short periods of breath-holding or over-breathing cause large alterations in the reaction.

Sugiura & Chesley (61) have found that transplantable tumour tissue can be soaked in Ringer's solution containing up to 40 per cent of heavy water for 24 hours without destroying its transplantability. It seems that, although there are some quantitative differences, not always very sharply defined, between the chemical constitution and behaviour of malignant and normal tissues, work of the type described in this section has not, as yet, given any definite clue to the solution of the cancer problem.

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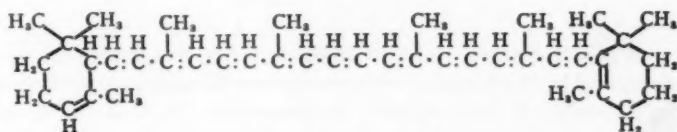
# PLANT PIGMENTS\*

By R. KUHN

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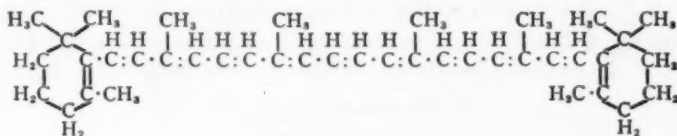
## CAROTENES AND CAROTINOIDS

*Hydrocarbons.*— $\alpha$ -Carotene, for the isolation of which calcium hydroxide is employed, possesses formula I, according to Karrer, Morf & Walker (1).



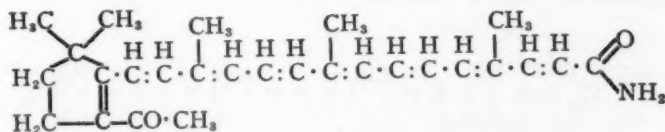
I.  $\alpha$ -Carotene,  $C_{40}H_{56}$

Karrer's formula for  $\beta$ -carotene, II, has been definitely established by means of degradation studies with chromic acid [Kuhn & Brockmann (2)].



II.  $\beta$ -Carotene,  $C_{40}H_{56}$

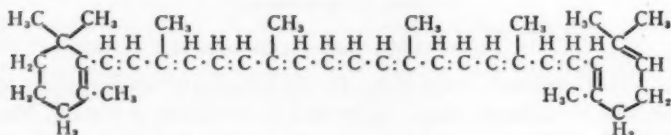
From the degradation products, dehydro-azafrinone amide, III (m.p. and mixed m.p. =  $209^{\circ}$ ), was prepared. This compound can also be prepared from azafrin, X.



III. Dehydro-azafrinone amide

\* Received January 28, 1935.

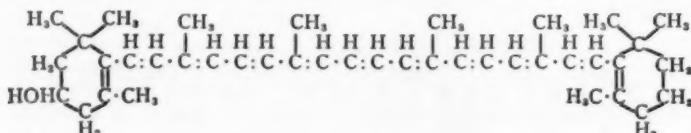
$\gamma$ -Carotene, discovered by Winterstein & Ehrenberg (3), constitutes approximately 0.1 per cent of the pigment of *Daucus carota*, and possesses formula IV [Kuhn & Brockmann (4, 5)].



IV.  $\gamma$ -Carotene,  $C_{40}H_{56}$

A new hydrocarbon of the carotinoid series,  $\delta$ -carotene, has been discovered by Winterstein (6) in the bark of *Gonocaryum pyriforme*.

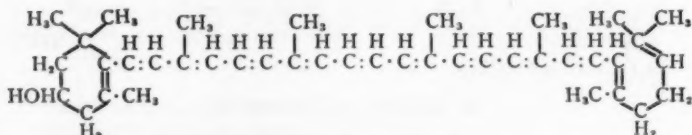
*Alcohols*.—A new pigment, kryptoxanthin, V,  $C_{40}H_{55}OH$ , has been found with zeaxanthin in the pods of *Physalis* (7), and in yellow corn [Kuhn & Grundmann (8)]. Although it contains an asymmetric carbon atom, the pigment is optically inactive.



V. Kryptoxanthin,  $C_{40}H_{55}OH$

According to Karrer & Schlientz (9) caricaxanthin from *Carica papaya* 1 [Yamamoto & Tin (10)] is identical with kryptoxanthin.

Rubixanthin, VI,  $C_{40}H_{55}OH$ ,  $[\alpha_D^{25} = \pm 0^\circ]$ , the simplest xanthophyll derivative of  $\gamma$ -carotene, IV, has been isolated from *Rosa rubiginosa* by Kuhn & Grundmann (11).



VI. Rubixanthin,  $C_{40}H_{55}OH$

Pure zeaxanthin is optically inactive (12). Another new xanthophyll, flavoxanthin ( $C_{40}H_{60}O_8$ , m.p.  $184^\circ$ ,  $[\alpha_D^{25}] = 190^\circ$ , benzene), has been isolated by Kuhn & Brockmann (13) from the petals of *Ranunculus acer*. The pigment yields an intense blue color with 25 per cent HCl.

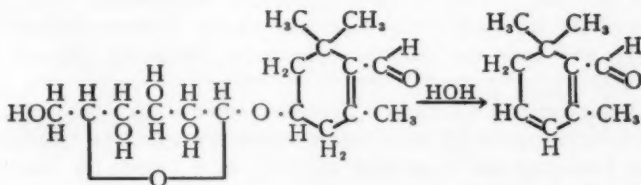
*Acids.*—A new pigment, isomeric with crocetin, VII, has been isolated from the stigma of *Crocus sativus* L (saffron) by Kuhn & Winterstein (14). It can readily be converted to crocetin by means of light, iodine, and other agents.



VII. Cis- and trans-crocetin,  $C_{20}H_{24}O_4$

This is an interesting case of cis-trans isomerism. The labile form of the dimethyl ester melts at  $141^\circ$  and absorbs at 458 and  $433.5 \text{ m}\mu$ ; the stable form of the dimethyl ester melts at  $222^\circ$ , and absorbs at 463 and  $434.5 \text{ m}\mu$  (chloroform).

The bitter agent of saffron, picrocrocin, is derived, according to Kuhn & Winterstein (15), from the ring system of a hypothetical "protocrocin," the oxidation of which in the plant yields one mol of crocetin ( $C_{20}$ ) and two mols of picrocrocin ( $C_{10}$ ), VIII. The picrocrocin later yields safranal, IX, and one molecule of *d*-glucose.

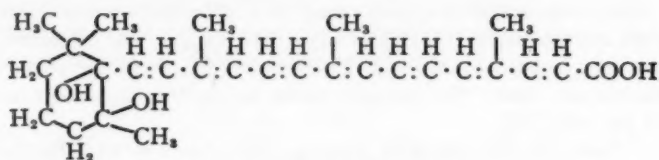


VIII. Picrocrocin

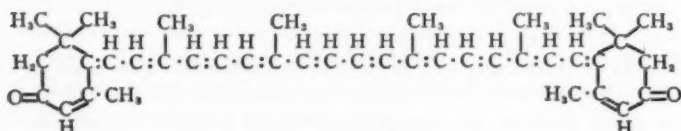
IX. Safranal,  
 $C_{10}H_{14}O$

Azafrin, from the roots of *Escobedia scabrifolia*, m.p.  $212^\circ$ ,  $[\alpha_D^{25}] = -75.3^\circ$ , ethyl alcohol] possesses formula X, according to Kuhn & Deutsch (16). It may, therefore, be regarded as a phytochemical degradation product of a carotinoid with 40 carbon atoms.



X. Azafrin,  $C_{27}H_{38}O_4$ 

**Ketones.**—Rhodoxanthin, XI, has been isolated from the red berry of the yew (*Taxus baccata*) by Kuhn & Brockmann (17) and identified as a diketone of the carotinoid series. Its absorption bands are displaced further toward the red than any other known carotinoid, and it is readily converted into a dihydro derivative.

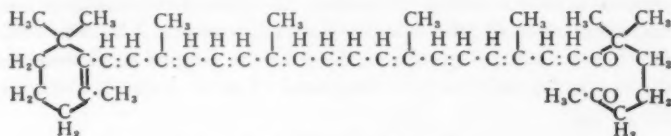
XI. Rhodoxanthin,  $C_{40}H_{50}O_2$ 

Mention should also be made of astacin, an animal pigment, which has been isolated in crystalline form from the shell, hypodermis, and eggs of the lobster, *Astacus gammarus* [Kuhn & Lederer (18)]. According to Karrer & Loewe (19), the pigment is a tetraketone,  $C_{40}H_{48}O_4$ , from which a dioxime and a diphenazine derivative have been prepared. Crystalline astacin has also been isolated from the eggs of the sea spider, *Maja squinado* [Kuhn, Lederer & Deutsch (20)], and from the starfish, *Ophidiaster ophidianus* [Karrer & Benz (21)].

Zechmeister & Cholnoky (22) have successfully analyzed the pigments of paprika by means of the chromatogram. The hydrocarbons,  $\beta$ -carotene and  $\alpha$ -carotene (traces), were found; the xanthophylls: kryptoxanthin, lutein, and zeaxanthin, and the specific pigments, capsanthin and capsorubin (a new xanthophyll), were also found. Capsorubin (507, 474, 444  $m\mu$ ) is hypophasic, and yields an epiphasic crystalline acetate.

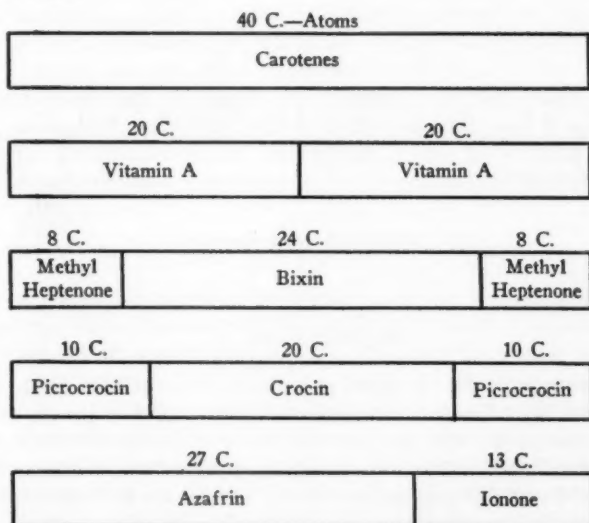
On the basis of recent researches the formula  $C_{40}H_{58}O_3 \pm 2H$  has been ascribed to capsanthin (m.p. 175–176°, absorption at 506 and 475  $m\mu$  in benzene). Two oxygen atoms are present in hydroxyl groups, which can be esterified. In the natural pigment, myristic,

palmitic, stearic, carnaubic, and oleic acids are present in esterified form. The third oxygen atom apparently exists as carbonyl in conjugation with the other double bonds of the molecule. In this connection Zechmeister & Chlcnoky point out the similarity of their pigment with semi- $\beta$ -carotenone, XII, of Kuhn & Brockmann (23), prepared by the mild action of chromic acid on  $\beta$ -carotene.



XII. Semi- $\beta$ -carotenone,  $C_{40}H_{56}O_2$

*Genetical relations.*—Theoretically, the cleavage of the carbon skeleton of a carotinoid with 40 carbon atoms yields other natural products, their structure depending upon the point of cleavage. It is quite possible that carotinoid derivatives are formed in some such manner in the living organism (24). This is shown diagrammatically as follows:

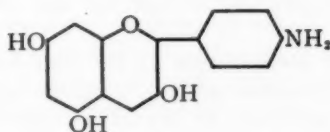


XIII. Diagram



glucoside by MacDowell, Robinson & Todd (32). 5-Glucosidyl-apigenin is identical with gesnerin from *Gesnera fulgens* (33). Likewise, synthetic malvidin-3-galactoside of Bell & Robinson (34) is identical with a pigment isolated from *Primula sinensis*. 3- $\beta$ -Glucosidyl-delphinidin chloride is in all probability identical with myrtillin from the whortleberry [Reynolds & Robinson (35)].

Of the various nitrogen-containing anthocyanins prepared by Robinson & Robinson (36), XVI exhibits the greatest similarity to betanin from beets.



XVI. Synthetic compound similar to betanin

The three anthocyanin types of Willstätter may occur simultaneously in one and the same plant, but in different parts of the plant. Thus, in a form of nasturtium, the petals contain pelargonidin, the pod a cyanidin, and the leaves a delphinidin derivative (27). The difference between the blue of the cornflower and the red of the rose is not dependent upon the pH of the cell sap (Robinson & Robinson). By a combination on cell colloids, which can be imitated *in vitro*, cyanin may remain blue even in an acid medium (cornflower).

#### THE CHLOROPHYLL PIGMENTS

*Bacteriochlorophyll*.—The pigment of the sulphur-free purple-bacteria, bacteriochlorin, has been characterized as a pigment related to, but not identical with chlorophyll [Noack & Schneider (37)]. In the organism and in ether solution, the pigment is violet, but upon the addition of alcohol or quinone, it changes into a green. Magnesium is removed by means of acids. The resulting phaeophorbide consists of two modifications, *a* and *b*, of which, in contrast to leaf-chlorophyll, the *b* modification,  $C_{38}H_{36}O_5N_4 \cdot \frac{1}{2}H_2O$  predominates. By means of HI-glacial acetic acid, a porphyrin mixture is obtained which spectroscopically resembles phyloerythrin. With Fe-formic acid, two por-

pyrins of the phaeoporphyrin type are obtained. Whether the original pigment contains phytol has not yet been established.

*Isolation of components a and b.*—Using the chromatographic adsorption technic of Tswett, Winterstein & Stein (38) have isolated the *a* and *b* components of leaf chlorophyll on a preparative scale. Cane sugar was used as the adsorptive agent. On calcium carbonate, allomerization took place. The pure components exhibit the following absorption bands in ether:

chlorophyll-*a*: 663, 623, 607, 577, 534, 507, 494, 423 m $\mu$

chlorophyll-*b*: 644, 614, 594, 567, 542, 503, 456, 428 m $\mu$

Exact photoelectric measurements of the absorption spectra were made, and a simple procedure given by which the relative amounts of the components *a* and *b* can be determined spectroscopically. The preparations of chlorophyll-*b* described by Willstätter, Stoll & Utzinger (39) and Willstätter & Stoll (40) were thus shown to contain about 10 per cent of chlorophyll-*a*. According to Stoll & Wiedemann (41) the earlier procedure for the separation of the components has been improved so much that it is also possible to prepare pure chlorophyll-*b* by this means. The most striking observation made is that the first absorption band in the red (39, 40) is not due to chlorophyll-*b*, but rather to the chlorophyll-*a* content of the preparation.

Chlorophyll-*c*, described by Zscheile (42), was not found in the nettle by Winterstein & Schoen (43). When the chromatographic analysis was improperly carried out, however, decomposition products resulted which might have been mistaken for chlorophyll-*c*.

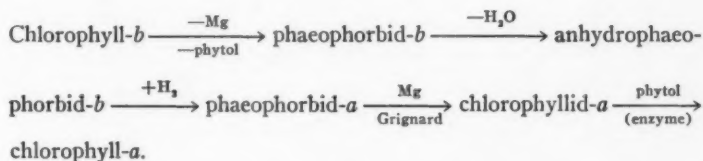
*Optical activity.*—The optical activity of chlorophyll preparations was determined photographically in a polarization apparatus, using light of wave length  $\lambda = 720$  m $\mu$  [Stoll & Wiedemann (44)]. The preparations rotated toward the left. The highest observed values for the rotation were as follows:

chlorophyll- <i>a</i>	$[\alpha]_{720}^{25} = -262^{\circ}$
phaeophytin- <i>a</i>	$[\alpha]_{720}^{25} = -126^{\circ}$
phaeophorbid- <i>a</i>	$[\alpha]_{720}^{25} = -342^{\circ}$
chlorophyll- <i>b</i>	$[\alpha]_{720}^{25} = -267^{\circ}$
phaeophytin- <i>b</i>	$[\alpha]_{720}^{25} = -133^{\circ}$
phaeophorbid- <i>b</i>	$[\alpha]_{720}^{25} = -316^{\circ}$

As solvents, acetone, or mixtures of methyl alcohol with acetone (10 per cent), or with pyridine (5 per cent), were used. On standing in solution, the optical activity was lost after eight days, although the results of the phase test were still positive. Racemization, therefore, was apparently independent of allomerization.

*Leucochlorophyll*.—According to Kuhn & Winterstein (45) chlorophylls *a* and *b* are readily reduced to brown "leucochlorophylls" by means of zinc, pyridine, and a small amount of glacial acetic acid. On exposure to air, the original green color and red fluorescence returns. The resulting product is identical with the starting material. The reversible reaction, chlorophylls  $\rightleftharpoons$  leucochlorophylls, may be of importance in the assimilation of carbon dioxide.

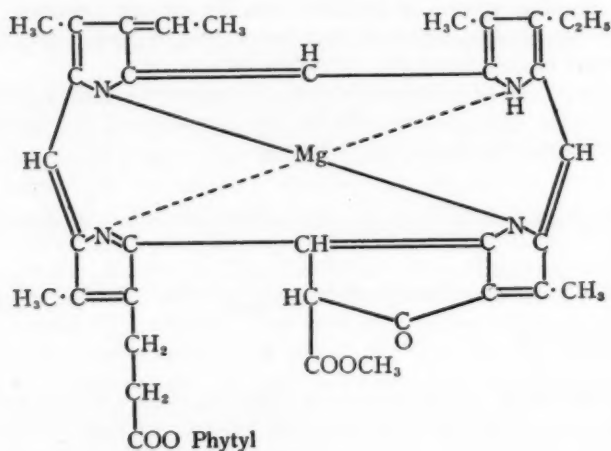
*Conversion of chlorophyll-*b* into chlorophyll-*a**.—Stoll & Wiedemann (46) claim to have effected the conversion of chlorophyll-*b* into chlorophyll-*a* in the following way:



*Function in assimilation*.—According to Stoll (47) photosynthesis can be divided into the following series of reactions: (a) Combination of carbonic acid with chlorophyll into a chlorophyll-carbonic acid complex; (b) transfer, similar to a peroxidative rearrangement, of the carbonic acid to hydrogen acceptors (photochemical reaction I); (c) dehydrogenation of the chlorophyll with a step-wise reduction of the carbonic acid; (d) cleavage of the water molecule attached to the chlorophyll,  $\text{H}_2\text{O} \rightarrow \text{H} + \text{OH}(\text{H}_2\text{O}_2)$ , the chlorophyll again becoming saturated with hydrogen (photochemical reaction II); (e) removal of the hydrogen peroxide by means of leaf catalase, an enzymatic reaction dependent upon temperature; release of oxygen. The possibility that chlorophyll is normally dehydrogenated and then hydrogenated in the leaf, as under the influence of quinone, was first suggested by Fischer *et al.* (48, 49). According to Stoll, the reverse process, hydrogenation followed by dehydrogenation, could take place (cf. leucochlorophylls). The considerations of Haber and Willstätter (50) suggest the possibility of the formation of radicle-like mono-

dehydro-chlorophylls in the photoreduction of carbonic acid [Willstätter (51)].

*Constitution of chlorophyll-a.*—The important researches of Fischer, which cannot be adequately reviewed in the space of this article, have led to the following formula for chlorophyll-*a* [Fischer & Hassenkamp (52)], in which the position of the Mg atom is as yet undetermined.



XVII. Chlorophyll-*a*

For methyl phaeophorbide, the formulation of a tautomeric form is also possible, in addition to the replacement of Mg by 2H, and phytol by methyl. Methyl chlorophyllide-*a* (positive phase test) was obtained in pure crystalline form by Fischer & Spielberger (53) who treated methyl phaeophorbide-*a* with Grignard reagent, and decomposed with alcohol in the presence of excess metallic magnesium and pyridine.

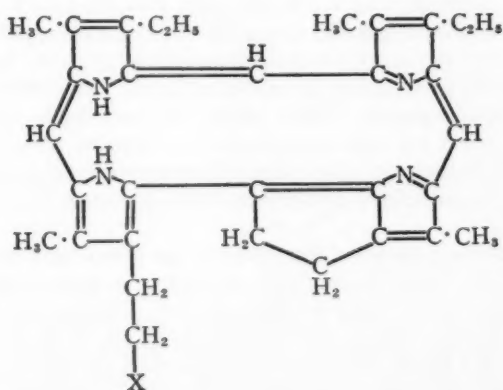
*Biological decomposition products of chlorophyll.*—The chief decomposition product isolated from elephant feces by Fischer & Hendschel (54) was phylloerythrin. In contrast to sheep feces, no



probophorbid was obtained. From human feces (vegetarians), probophorbid, m.p.218°, was obtained. Decarboxylation of the chlorophylls appears to be the characteristic reaction in the digestive tract. Using Fischer's method for the isolation of phyllobombicin, Marchlewski & Urbańczyk (55) isolated anhydrophyllotaonin from the feces of the silkworm. This compound may be identical with phaeopurpurin-18 (phyllopurpurin).

From benzoate stones, Treibs (56) isolated phylloerythrin (0.02 per cent). In a stone from the digestive tract of the horse he found two probophorbids in addition to two pigments: lithoporphyrin (perhaps a precursor of phylloerythrin) and lithochlorin. Lithochlorin is green and exhibits an absorption spectrum of five bands.

*Chlorophyll and hemin derivatives in bituminous earths, mineral oils, and asphalts.*—Chlorophyll and hemin derivatives have been found in bituminous earths, mineral oils and waxes, and in asphalts by Treibs (57). Desoxophyllerythrin, XVIII, and desoxophyllerythro-aetioporphyrin, XIX, were isolated in considerable amounts from oil schists (Alpine Trias).



XVIII. (X = COOH) Desoxophyllerythrin  
XIX. (X = H) Desoxophyllerythro-aetioporphyrin

In addition mesoporphyrin and meso-aetioporphyrin were identified (derivatives of hemin). Hence, all theories of the origin of petroleum

which involve distillation must be abandoned. It appears established that plants and animals were involved in the formation of petroleum, with plants playing a major rôle. The processes which led to the separation of these products in the earth may be compared to the action of a huge chromatogram (Tswett).

*Phycobilins of red algae.*—The phycobilins of red algae are produced [Lemberg & Bader (58)] either from the hydrogenation of cytochrom-hemin or from a side reaction in the synthesis of chlorophyll. From phycocyan and phycoerythrin they obtained dehydromesobilirubin (glaucoobilin) through the action of 10 per cent potassium hydroxide in methyl alcohol; from phycoerythrin, mesobilirubin was obtained in addition. This definitely places the pigments of the red algae in the bile-pigment series.<sup>1</sup>

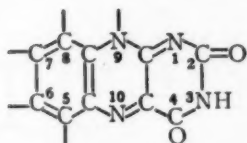
### FLAVINS<sup>2</sup>

Lactoflavin [Ellinger & Koschura (59); Kuhn, György & Wagner-Jauregg (60)],  $[C_{17}H_{20}N_4O_6]$ , m.p. 287°,  $[\alpha]_D = -115^\circ$  in 0.05N NaOH], first obtained from milk, also has been isolated from green leaves by Kuhn & Kaltschmitt (61), and thus recognized as a plant pigment. A plant origin was first suggested by the demonstration that a vitamin (G, B<sub>2</sub>) is present in lactoflavin [György, Kuhn & Wagner-Jauregg (62)]. In green leaves the molecular ratio, flavin: chlorophyll, is approximately 1:2000 (61). The distribution of flavin in the plant world has been summarized in a table by Kuhn, Wagner-Jauregg & Kaltschmitt (63). Crystalline flavins have also been prepared by Karrer & Schoepp from dandelion blossoms (64) and from malt (65).

By irradiating the prosthetic group of the yellow enzyme of yeast, a chloroform-soluble flavin,  $C_{13}H_{12}N_4O_2$ , was first prepared in crystalline form by Warburg & Christian (66). Apparently the same pigment, lumi-lactoflavin, was obtained by Kuhn, Rudy & Wagner-Jauregg (67) by the irradiation of lactoflavin in alkaline solution. Degradation and model experiments in the alloxazine series [Stern & Holiday (68)] suggested the following formula for the chromophore XX [Kuhn & Rudy (69)]:

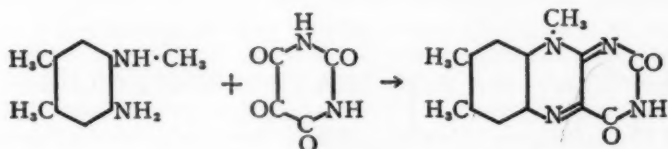
<sup>1</sup> Cf. Fischer, H., and Orth, H., *Ann. Rev. Biochem.*, **3**, 410 (1934)

<sup>2</sup> See also this volume, pp. 17, 32, 338. (EDITOR.)



XX. Flavin = iso-alloxazine

The constitution of lumi-lactoflavin,  $C_{13}H_{12}N_4O_2$ , was definitely established by synthesis as 6,7,9-trimethyl-flavin [Kuhn, Reinemund & Weygand (70)]. The condensation of *N*-substituted aromatic *o*-diamines with alloxan [Kuhn & Weygand (71)] is a general reaction. For the synthesis of lumi-lactoflavin (XXIII), 1,2-dimethyl-4-amino-5-methylamino-benzol (XXI) was condensed with alloxan (XXII).

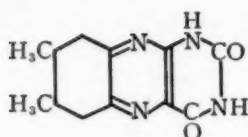


XXI. 1,2-Dimethyl-4-amino-5-methylamino benzol

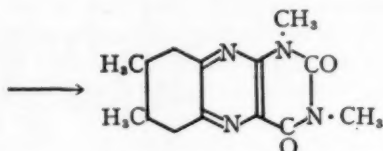
XXII. Alloxan

XXIII. Lumi-lactoflavin = 6,7,9-trimethyl-flavin

As much pigment can be obtained from 100 gm. of diamine XXI as from 750,000 liters of milk. By irradiating lactoflavin in neutral solution Karrer, Salomon, Schoepp, Schlittler & Fritzsche (72) obtained lumichrome,  $C_{12}H_{10}N_4O_2$  [6,7-dimethyl-alloxazine (XXIV)]. Its constitution was definitely established by Kuhn & Rudy (73) by methylation with diazomethane, whereby 1,3,6,7-tetramethylalloxazine, XXV, was obtained, identical with the condensation product (74) of 1,2-dimethyl-4,5-diamino-benzol with dimethylalloxan (m.p. and mixed m.p. 252°).

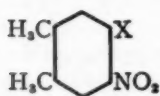
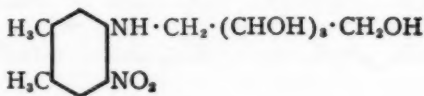


XXIV. Lumichrome



XXV. 1,3,6,7-Tetramethylalloxazine

The methyl imide group of lumi-lactoflavin, XXIII, is formed during the alkaline photolysis of lactoflavin (67, 69) which contains four more carbon atoms and four more hydroxyl groups (acetylation) than lumi-lactoflavin. Apparently the tetra-oxybutyl residue,  $-\text{CHOH}\cdot\text{CHOH}\cdot\text{CHOH}\cdot\text{CH}_2\text{OH}$  [Kuhn, Rudy & Wagner-Jauregg (67)], is attached to a  $\text{CH}_2$  group on N-atom 9. Compounds of this type have been synthesized by Kuhn & Weygand (75) by reducing pentose oximes to the corresponding amines, and condensing with compounds of type XXVI.

XXVI.  $\text{X} = \text{Cl}, \text{Br},$   
 $\text{I}, \text{NO}_2 \text{ or}$   
 $\text{OSO}_2 \cdot \text{C}_6\text{H}_5$ 

XXVII. Tetrahydroxy-amyl-dimethyl-nitraniline

The resulting tetrahydroxy-amyl-nitranilines, XXVII, were reduced to the diamines, and condensed with alloxan. The pigment obtained with *l*-arabinose promotes growth in rats on a diet deficient in vitamin  $\text{B}_2$  (0.015 mg. per day), and shows a similar rotation ( $-110^\circ$ ) as lactoflavin ( $115^\circ$ ) [Kuhn & Weygand (76, 77)]. 6,7-Dimethyl-9-*l*-araboflavin, XXVIII, is therefore the first synthetic compound showing vitamin- $\text{B}_2$  activity. Since this compound, like lactoflavin, is apparently converted by the organism into a "yellow enzyme" [Warburg & Christian (78)], it may be considered the first synthetic active group of an enzyme.



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## THE ALKALOIDS\*

BY ROBERT ROBINSON

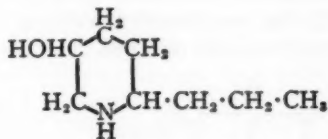
*The Dyson Perrins Laboratory,  
Oxford University*

Progress in the elucidation of the molecular structure of the alkaloids has been steady but not spectacular during the past two years. No outstanding developments fall to be recorded in connection with the major problems, such as morphine or strychnine, but there has nevertheless been great activity in these fields as well as in relation to the less fully investigated natural bases which often offered less resistance to the chemist's curiosity.

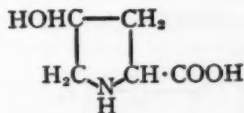
The various subjects are briefly mentioned below under the headings of the names of individual alkaloids. Certain topics, notably stereochemical relations, could not be included owing to limitations of space.

*ψ-Conhydrine*.—Of the five alkaloids of hemlock (*Conium maculatum*) this was the only one of undetermined constitution and the gap has now been filled by Späth and his collaborators (1). The secondary base,  $C_8H_{17}ON$ , contains an alcoholic hydroxyl and there was already evidence for the probable assumption that we have to deal with a hydroxyconiine; the position of the hydroxyl was unknown.

The methine was catalytically hydrogenated to  $C_{10}H_{23}ON$  and then oxidised by potassium permanganate with formation of *n*-heptoic acid. The only reasonable interpretation of this result is that *ψ*-conhydrine possesses the structure given by formula I.



I. *ψ*-Conhydrine



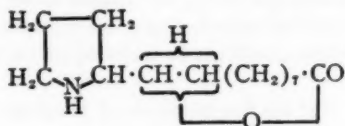
II. Hydroxyproline

It may be pointed out that this site of the hydroxyl group is of interest in connection with the parallelism assumed by the reporter between piperidine and pyrrolidine bases in relation to their origin from lysine

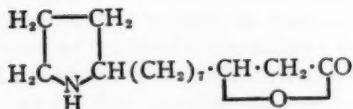
\* Received March 22, 1935.

and ornithine respectively. I may be compared with hydroxyproline (II). The constitution of conhydrine as 2- $\alpha$ -hydroxypropylpiperidine has been confirmed by the results of Hofmann degradations, reductions of the methines and oxidation of the products (2).

*Carpaine*.—This secondary base,  $C_{14}H_{25}O_2N$ , is readily hydrated with formation of an amino-hydroxy-acid (carpamic acid),  $C_{14}H_{27}O_3N$ . Careful oxidation of carpamic acid by means of permanganate or nitric acid affords suberic acid, mixed in the latter case with azelaic acid. It would thus appear that  $-C(CH_2)_7C-$  must be a unit of the structure. Taken in conjunction with other evidence of the occurrence of an  $\alpha$ -substituted pyrrolidine fragment, this indicates that the lactone-ring of carpaine is either unusually small or unusually large and formulae of the types III and IV come into question (3).

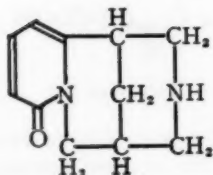


III. Carpaine (?)

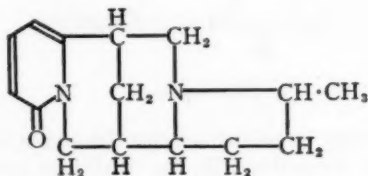


IV. Carpaine (?)

*Anagyrene*.—The constitution of cytosine established in the later phases of the research by Späth and by Ing was discussed in the report for 1932;<sup>1</sup> Ing's formula (V) has not been since modified.



V. Cytosine



VI. Anagyrene (?)

<sup>1</sup> Robinson, R., *Ann. Rev. Biochem.*, 2, 421 (1933).

It has been used to suggest a working hypothesis in regard to the structure of the very interesting congener of cytosine (in *Anagyris foetida*) termed anagryne (4). The importance of the subject was much enhanced by the observations that catalytic reduction of anagryne affords *l*-lupanine (tetrahydroanagryne) while electrolytic reduction yields *d*-sparteine (hexahydrodesoxyanagryne). In this connection it may be recalled that Clemo, Raper & Tenniswood converted *l*-lupanine into *d*-sparteine by reduction with hydriodic acid and phosphorus (5). Anagryne is thus a link between cytosine and sparteine and it probably bears a relation to cytosine similar to that which sparteine bears to lupinine. The composition difference between anagryne and cytosine is  $C_4H_6$ , which suggests an additional ring, represented in VI as a pyrrolidine ring, but there is an alternative formula with a new piperidine ring [obtained from VI by writing :  $CH(CH_3)$  in the ethylene form  $\cdot CH_2 \cdot CH_2 \cdot$ ].

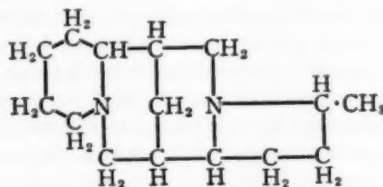
On oxidation with barium permanganate anagryne yields only one cyclic amide, namely anagryamide,  $C_{15}H_{18}O_2N_2$  (by :  $N \cdot CH_2 \cdot \rightarrow : N \cdot CO \cdot$ ), and the action of ozone on this substance affords a lactam,  $C_{11}H_{16}O_2N_2$ . The degradation is exactly the same as that known in the cytosine group and it involves the removal of the  $\alpha$ -pyridone nucleus.

Furthermore, the position of the new carbonyl group of anagryamide is probably  $\beta$ - to the pyridine nucleus because anagryamide loses carbon dioxide when it is heated with hydriodic acid and phosphorus to  $240^\circ$ . The analogies cited here are with the behaviour of *N*-methylcytisamide and with that of 6-hydroxy-4-methylpyridine-2-acetic acid, the latter losing a carboxyl group on fusion (6).

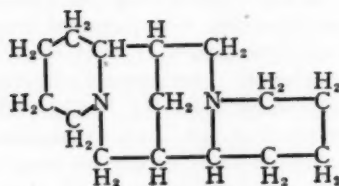
These facts give a plausible basis for the proposed position of attachment of the last ring, as in VI, and further information was obtained by the study of the degradation of anagryne by exhaustive methylation combined with reduction of the methines. At the third stage trimethylamine was eliminated and the reduced product,  $C_{15}H_{23}ON$ , was ozonised with consequent destruction of the hitherto unscathed pyridone nucleus. The resulting cyclic amide,  $C_{11}H_{21}ON$ , was hydrolysed and oxidised to  $\alpha$ -methyl- $\alpha'$ -*n*-amylglutaric acid. This result is strongly confirmatory of VI (or the piperidine-type isomeride already mentioned) and the related sparteine formulae are therefore VII and VIII (p. 500).

The subject is not yet free from dubiety but Ing is to be congratulated on the extent of the progress resulting from his investigations of

cytisine and anagryne. In doing this we do not forget the significant results of Ewins, Karrer, Späth, and Clemo among others.



VII. Sparteine (?)



VIII. Sparteine (?)

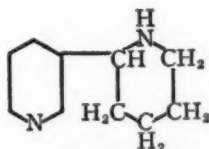
Finally it may be mentioned that the sparteine of *Cytisus scoparius* is *l*-sparteine and that the *d*-sparteine obtained from *l*-lupanine has now been found in *Sophora pachycarpa* (7) and in *Thermopsis lanceolata* (8).

The structural relations of the various alkaloids of the pyridine-piperidine group are clear enough and the assumption of an origin from lysine or from some progenitor of lysine is a very attractive hypothesis. It does not seem justifiable, however, to speculate on the actual mechanism of the phytochemical synthesis in detail. Everything that we know about the course of reactions *in vivo* shows that the reality is complex whereas our tendency is to produce paper syntheses as simple as possible. The reporter would like to use this opportunity to emphasise once again that we can often recognise the structural units of the molecular edifice but the only way to find out how the sections are fitted together is by experimental work in plant biochemistry and physiology. Thus the suggestions of Schöpf, Schmidt & Braun in regard to the phytosynthesis of lupanine (9) are considered to be of value only in so far as the two lysine residues are

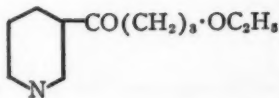
recognised, the actual synthetic reaction formulated being possible but not supported by evidence.

*Anabasine*.—Orekhov & Menschikov (10) have isolated this isomeric of nicotine,  $C_{10}H_{14}N_2$ , along with lupinine and three other alkaloids from *Anabasis aphylla* (Chenopodiaceae), a native of Central Asia. The anthocyanins of many Chenopodiaceae belong to the nitrogenous group typified by betanin, but alkaloids have not previously been encountered in this natural order.

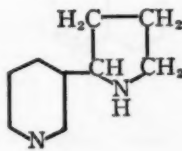
Anabasine is levorotatory and on dehydrogenation with silver acetate or zinc dust it yields 2,3'-dipyridyl, identified with a synthetic specimen; the base therefore possesses the structure given by formula IX. As a pyridine derivative it may be aminated by means of sodamide and on reduction it furnishes an optically active dipiperidyl



IX. Anabasine



X



XI. Nornicotine

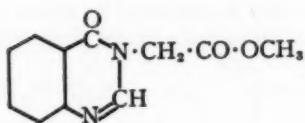
among other substances. It seems certain that anabasine has the constitution IX but at one stage the question arose as to a possible ring-enlargement from a methylpyrrolidine. The very useful Kuhn-Roth method of estimation of side-chain methyl groups by oxidation to acetic acid could be applied to the rapid solution of the problem, which, as indicated above, is also encountered in the anagyrine-sparteine group. Anabasine finds application as an insecticide and it has been shown that *dl*-anabasine is obtained along with other substances when the reaction product of sodium dipyridyl and pyridine is aërated (11).

Craig (12) has developed a synthesis of *nornicotine* which together with anabasine constitutes the so-called *nicotine* obtainable from Kentucky tobacco (13). Nicotinonitrile and  $\gamma$ -ethoxypropyl magnesium bromide gave the ketone X, the oxime of which was reduced to a primary amine and then heated with aqueous hydrobromic acid forming *nornicotine* (XI).

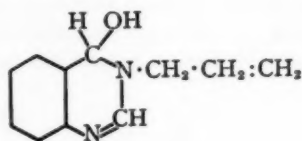
This is perhaps a convenient place to mention the synthesis (14) of  $\alpha$ -nicotine, 2-(2'-pyridyl)-1-methylpyrrolidine, by reduction of  $\alpha$ -nicotyrine with zinc, hydrochloric acid, and a little platinic chloride.

The study of such analogues of nicotine is probably connected with the search for insecticides.

*Vasicine (Peganine).*—The doubts expressed in the previous report<sup>2</sup> respecting the interpretation of the reactions of this base have been justified by recent work. The interest attaching to the base has meanwhile been increased by its isolation from the mother liquors of alkaloids from *Peganum harmala* (15). On oxidation with permanganate, followed by treatment of the product with diazomethane, methyl-4-keto-3,4-dihydroquinazoly-3-acetate (XII) was produced and fully identified.



XII



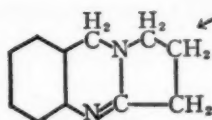
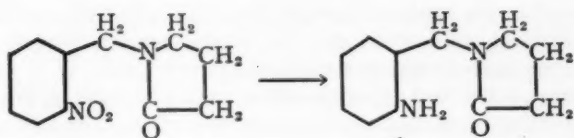
XIII

Hence Späth & Nikawitz suggested that peganine (vasicine) has the constitution XIII, although other possibilities were mentioned. The preferred formula is not in agreement with the failure to reduce vasicine by catalytic methods or with the formation of a chlorodesoxyvasicine by the action of phosphoryl chloride on the base. XIII is a carbinol amine and such treatment should convert it into a quinazolinium salt. The synthesis of XIII (16) by the action of potassium hydroxide on allylquinazolinium iodide in cold aqueous solution proved it to be quite different from vasicine. Confirmation was found (17) in the non-identity of synthetic 3-allyl-1,2,3,4-tetrahydroquinazoline with dihydrodesoxyvasicine obtained by reduction of the base with sodium and amyl alcohol.

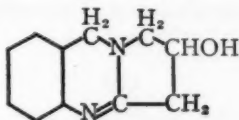
In a very recent paper Späth, Kuffner & Platzer (18) recognised the inadequacy of XIII and gained solid ground by effecting a synthesis of desoxyvasicine (XIV). *o*-Nitrobenzyl chloride and methyl  $\gamma$ -aminobutyrate afforded a pyrrolidone derivative which was reduced by stannous chloride to the related amine. Ring-closure by means of phosphoryl chloride produced desoxyvasicine, obtainable also from vasicine by replacement of hydroxyl by chlorine and reduction of the product.

<sup>2</sup> Robinson, R., *Ann. Rev. Biochem.*, 2, 421 (1933).

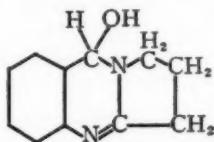




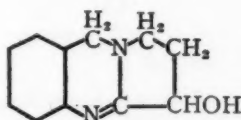
XIV. Desoxyvasicine



XV. Vasicine (?)



XVI. Vasicine (?)



XVII. Vasicine (?)

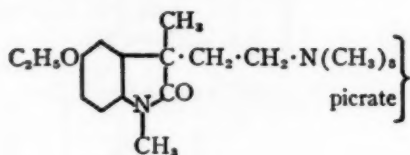
Professor J. N. Rây (private communication, 1934) has also obtained synthetic evidence favouring the skeleton of XIV for vasicine. The alkaloid is therefore XV or XVII, the constitution XVI, left in the running by Späth, being excluded by the properties of chlorodesoxyvasicine and also rendered improbable by the ready oxidation with permanganate. Of XV and XVII, the tendency appears to be to favour XV but the reviewer prefers XVII in view of the occurrence of vasicine in nature in the racemic form. The formulae explain the oxidation results equally satisfactorily, for in either case a group,  $-\text{CH}_2 \cdot \text{CO}-$ , would be first formed and then oxidised to two carboxyl groups. Späth and his colleagues cite an analogy for the spontaneous decarboxylation of the intermediate dibasic acid.

It is instructive to note the occurrence in vasicine of a chain of four carbon atoms terminated by nitrogen atoms, that is the ornithine chain; vasicine is a congener of alkaloids of the indole group and these are related to tryptophane which also contains the ornithine skeleton.

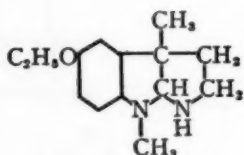
*Lysergic acid and ergine.*—The hydrolysis of ergotoxine and ergotinine by means of alcoholic potash furnishes the base ergine, the composition of which is now given as  $\text{C}_{16}\text{H}_{17}\text{ON}_8$  and which is now

recognised as the amide of lysergic acid,  $C_{14}H_{12}N(NCH_3) \cdot COOH$  (19). If, as at one time thought, this is an indole derivative, it must contain two further rings or a ring and a double bond.

*Eserine*.—The final establishment of the constitution of this base by the synthetical method is not far removed. The occurrence of the angle methyl group was demonstrated by the synthesis and resolution of the derivative XVIII (20) and now a further step has been taken by the resolution of *dl*-noreserethole (XIX) first synthesised by Robinson & Suginome (21).



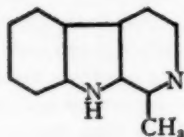
XVIII

XIX. *dl*-Noreserethole

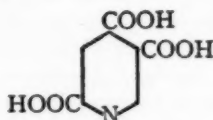
This advance is due to Hoshino and his collaborators. The active base yielded eserethole methiodide on treatment with methyl iodide (22).

The synthesis of a crystalline eserethole is described (23) but this could not be resolved and it appears to be a stereoisomeride of true *dl*-eserethole.

*Yohimbine*.—This base is the chief alkaloid of *Corynanthe Jorhimbe* (Rubiaceae) in which it occurs along with five isomerides. The substance is the methyl ester of yohimbic acid,  $C_{19}H_{23}ON_2 \cdot COOH$ , and on decarboxylation of the latter an alcohol,  $C_{19}H_{24}ON_2$ , termed yohimbol, is obtained. Passing over earlier observations because they are covered by the later work, a great advance in our knowledge is due to the recognition by Barger & Scholz (24) that a base " $C_{13}H_{12}N_2$ ," obtained by Warnat (25) is really  $C_{12}H_{10}N_2$  and is identical with harman (XX). This base is obtained by fusing yohimbic acid with potassium hydroxide or by heating it with zinc dust.

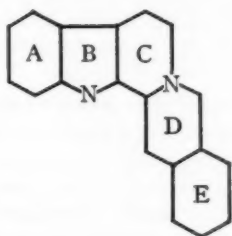


XX. Harman

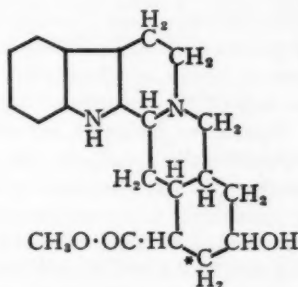


XXI. Berberonic acid

When the alkaloid is heated with selenium it affords yobyryne,  $C_{19}H_{16}N_2$  (26), tetrahydroyobyryne,  $C_{19}H_{20}N_2$ , and ketodihydroyobyryne,  $C_{20}H_{16}ON_2$ . The latter affords 2,3-dimethylbenzoic acid on fusion with alkali (Mendlik & Wibaut). Barger & Scholz identify *norharman* as a second product of the decomposition. These authors have also isolated berberonic acid (XXI) from the products of the oxidation of tetrahydroyobyryne with nitric acid. These results lead to the skeleton formula XXII to which a carboxyl group must be added.



XXII. Skeleton of yohimbine



XXIII. Yohimbine (?)

Berberonic acid is derived from ring D; rings D and E survive in the *isoquinoline* obtained in small yield by heating yohimbic acid with zinc dust (27). Yohimbine or yohimbic acid affords *m*-toluic acid when heated with superheated steam and a possible formula is shown in XXIII<sup>3</sup> although the position of the hydroxyl group is uncertain and it may occupy the asterisked position. A smooth decomposition is described by Hahn and co-workers (28). Yohimbine (1 mol) and lead tetra-acetate (2 mols) yield tetrahydroyohimbine hydrolysed to tetrahydroyohimbic acid,  $C_{19}H_{16}ON_2 \cdot COOH$ . The latter furnishes harman and *m*-toluic acid when heated with amyl alcoholic potassium hydroxide.

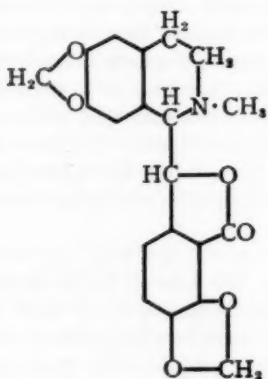
*Papaverine*.—Ingenious modifications of the synthesis have been described (29). Acetoveratrone is heated with sulphur and dimethylamine at  $145^\circ$  to give homoveratrodimethylthioamide in a yield of 65 per cent. This is hydrolysed by means of potassium hydroxide (yield of 84 per cent) and when heated in boiling tetralin under hydrogen

<sup>3</sup> The formula is misprinted in *Ber.*, 67, 690 (1934) (EDITOR).

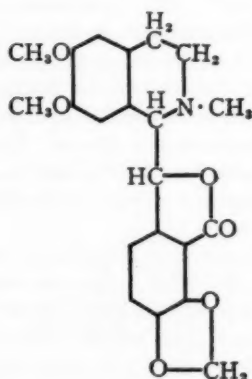
with  $\beta$ -veratrylethylamine, the yield of the amide is 96 per cent. Cyclisation to dihydropapaverine is effected by means of phosphoryl chloride in benzene solution and the yield is quantitative. Finally, dehydrogenation of dihydropapaverine is carried out by means of palladium black in boiling dihydrophenanthrene (yield of 86 per cent).

*Narcotine and hydrastine*.—Partial racemisation or epimerisation of the phthalide asymmetric carbon atom of these bases occurs under the influence of hot methyl alcoholic potassium hydroxide and whereas narcotine (*l*- $\alpha$ -narcotine) furnishes an isomeride of lower rotatory power (*l*- $\beta$ -narcotine), hydrastine (*l*- $\beta$ -hydrastine) affords one of higher rotatory power (*l*- $\alpha$ -hydrastine) (30). Although  $\beta$ -gnoscopine (*dl*- $\beta$ -narcotine) has not been resolved, it was found possible to synthesise it by mixing *l*- $\beta$ -narcotine with *d*- $\beta$ -narcotine (from *d*- $\alpha$ -narcotine derived by resolution of  $\alpha$ -gnoscopine).

*Bicuculline, adlumine, and bicucine*.—The phthalide group of the isoquinoline alkaloids has at last provided fresh alkaloidal material and Manske (31) has discovered three new examples of the type among the constituents of fumaraceous plants. Bicuculline is one of the bases isolated from *Dicentra cucullaria* and it is rather widely distributed, being also found in *Corydalis sempervirens*, *Adlumia fungosa* Greene and probably other plants. On oxidation the dimorphic alkaloid (m.p. 177° and 196°) yields hydrastinine and 2-carboxy-3,4-methylenedioxybenzaldehyde and as this fission is characteristic the constitution must be XXIV.

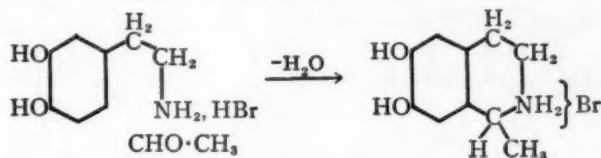


XXIV. Bicuculline



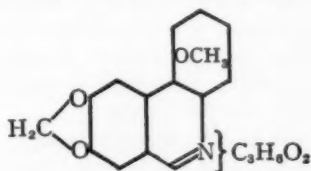
XXV. Adlumine

It is curious that the lactone ring in bicuculline appears to be less stable than in narcotine or hydrastine because a base isolated from several of the plants examined, and termed bicucine, appears to be the hydroxy acid corresponding with XXIV. In boiling dilute hydrochloric acid an equilibrium between lactone and hydroxy acid is established. Adlumine (31) from *Adlumia fungosa* must be represented as XXV because on oxidation with dilute nitric acid it yields 6,7-dimethoxy-2-methyl-3,4-dihydroisoquinolinium nitrate and 2-carboxy-3,4-methylenedioxybenzaldehyde. In connection with the biogenesis of isoquinoline alkaloids the reaction illustrated below has been examined and found to proceed readily at pH 5 and 25° (32).

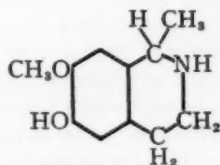


Similar experiments are recorded by Hahn & Schales (33), who appear to have overlooked their anticipation by Späth & Berger (34). A useful collation of the relations of the isoquinoline alkaloids was contributed by Barger to the International Conference of Pure and Applied Chemistry (Madrid, 1934).

*Tazettine*.—This interesting alkaloid,  $C_{18}H_{21}O_5N$ , is obtained from the dried corms of *Narcissus Tazetta* L. It contains a methoxyl and a methylenedioxy group and, on distillation with zinc dust, yields phenanthridine. Oxidation with permanganate furnishes hydrastic acid (4,5-methylenedioxyphthalic acid). Hofmann degradation results in a methine (with loss of methoxyl and formation of a new aromatic ring) and a second stage gives trimethylamine and 6-phenylpiperonyl alcohol which was synthesised. Tazettine is thus to be represented by the partly developed formula XXVI (35). It is possible that tazettine is identical with previously described bases, e.g., sekisanine.



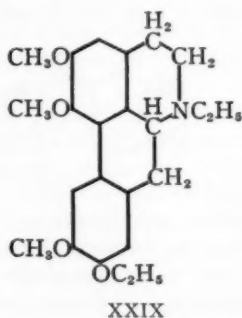
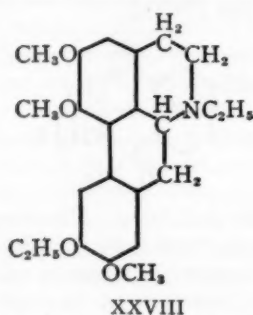
XXVI. Tazettine



XXVII. Salsoline

Work on the alkaloids of the Cactaceae has been prosecuted vigorously but the results offer no important novelty. A passing reference may be made, however, to salsoline found in *Salsola Richteri* (36), which by synthesis (37) is proved to be XXVII. Its N-O-dimethyl derivative is carnegine.

*Aporphine group.*—The constitution of laurotetanine has been determined (38) as the result of the synthesis of 2,5,6-trimethoxy-3-ethoxy-N-ethylnoraporphine (XXVIII) and 3,5,6-trimethoxy-2-ethoxy-N-ethylnoraporphine (XXIX) following the methods which have become almost standardised in this group.

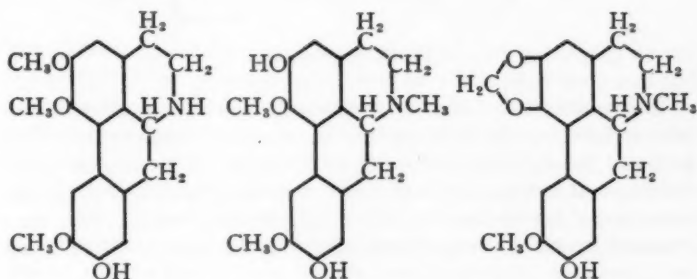


The optically active ON-diethylaurotetanine cannot be compared directly with the synthetic isomerides but this is feasible after submission to a process that destroys the dissymmetric character of the molecule. The methines derived from XXIX and from ON-diethylaurotetanine were identical and different from that obtained from XXVIII; identical products were also obtained from XXIX and from ON-diethylaurotetanine on ring fission by means of ethyl chloroformate (or benzoyl chloride). Laurotetanine, therefore, may be represented by formula XXX, opposite.

N-methylaurotetanine has been isolated from *Litsea citrata* (39) and its constitution demonstrated by ethylation followed by degradation along known lines to 3,4,6-trimethoxy-7-ethoxyphenanthrene. What may be described as an *isovanillin* type of orientation appears to predominate in the isoquinoline group and it is interesting to note that codeine and scinomenine contain similar structural units.

A substance which contains the unit in both aromatic sections of the aporphine structure is boldine (XXXI); it will be noted that the methoxyl group is para to the amino-acid side-chain in each residue of

the hypothetical *protoboldine*. In this case the necessary synthetic work and degradation of the diethyl ether of the natural product has been accomplished by Späth & Tharrer (40) and simultaneously by Schlittler (41) and with the same results. The former authors synthesised the appropriate dimethoxydiethoxyphenanthrene whereas Schlittler synthesised *r*-boldine diethyl ether and showed that in ring scission it yielded products identical with those derived from natural boldine diethyl ether.



XXX. Laurotetanine

XXXI. Boldine

XXXII. Actinodaphnine

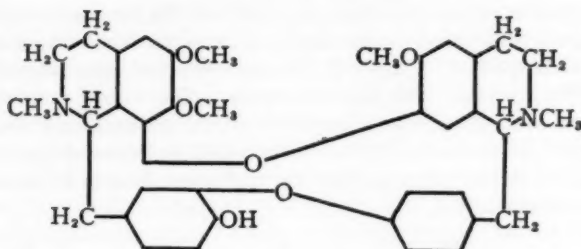
Actinodaphnine (XXXII) is a methylenedioxy analogue of laurotetanine; its constitution has been established by Hofmann degradation of its O-ethyl-N-methyl derivative followed by hydrolysis of the methylenedioxy group (sulphuric acid and phloroglucinol) and its replacement by two methoxyl groups. The laurotetanine series was then entered.

*Di-isoquinolines*. — The structure assigned to oxyacanthine (XXXIII) has been confirmed by Bruchhausen and his co-workers (43). The doubtful remaining points<sup>4</sup> were concerned with the orientation of certain methoxyl groups and of the diphenyl ether group in the upper section of the molecule as printed (p. 510).

These secrets would be disclosed if the constitution of a dialdehyd-trimethoxydivinyldiphenyl ether obtained by exhaustive methylation could be determined. The substance has now been reduced in stages to 2,3,2'-trimethoxy-6,5'-dimethyl-5,4'-diethyldiphenyl ether (XXXIV) which has been synthesised by applying Ullmann's reaction to the coupling of 2-bromo-3,4-dimethoxy-6-ethyl toluene and 4-methoxy-6-ethyl-*m*-cresol.

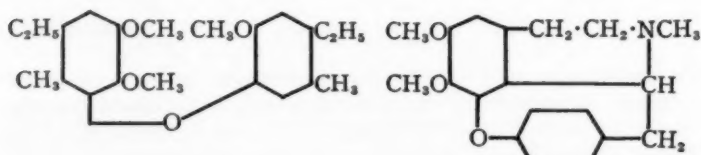
<sup>4</sup> Cf. *Ann. Rev. Biochem.*, 2, 440 (1933).





XXXIII. Oxyacanthine

Berbamine methyl ether and tetrandine appear to be stereoisomerides and structurally different from oxyacanthine methyl ether. Oxidation of the methines of the former by means of ozone yields 5,4'-dialdehydo-2-methoxydiphenyl ether and 6,5'-dialdehydo-2,3,2'-trimethoxy-5,4'-bis-( $\beta$ -dimethylaminoethyl)-diphenyl ether. This confirms the structure already suggested for berbamine. It will be noted



XXXIV

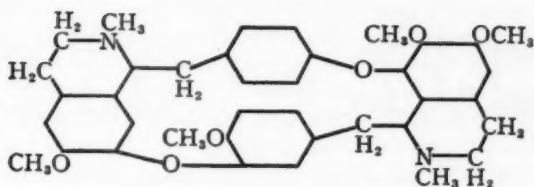
XXXV

that berbamine methyl ether and oxyacanthine methyl ether give different methines but identical oxidation products thereof and that the isomerism of the bases is due to a cause best represented by the schemes:



When the unions represented by vertical lines are dissolved identical products are obtained. This is, for example, realised when the methines are oxidised.

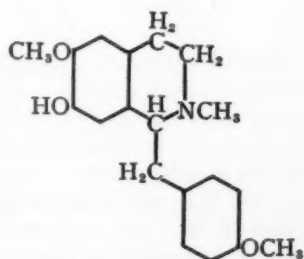
For *l*-curine from tubocurare, Späth, Leithe & Ladeck (44) suggested formula XXXV but later work (45) showed that this enantiomorph of *d*-bebeerine has the double formula XXXVI.

XXXVI. *d*-Bebeerine

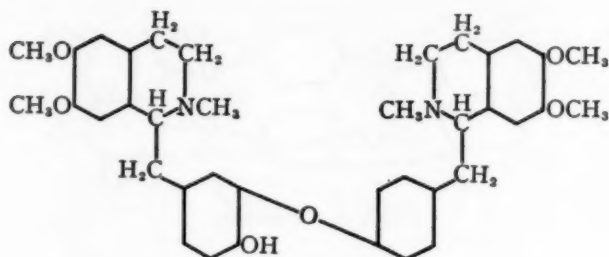
The formula is not free from dubiety; it is based essentially on the recognition of the base as a member of the di-isoquinoline group and on the earlier results of degradations, especially the formation of *p*-hydroxybenzoic acid and protocatechuic acid on fusion with alkali, and the production of 5,6,4'-tricarboxy-2,3-dimethoxydiphenyl ether by oxidation of the end-product of the Hofmann eliminations.

Another alkaloid originally thought to be a simple isoquinoline and which may be a di-isoquinoline is dauricine, for which Kondo & Narita, who isolated it from *Menispermum dauricum*, suggested formula XXXVII (46). Since a diphenyl ether derivative is obtained by exhaustive methylation and oxidation of the product it seems that the low molecular weight observed in fused camphor (Rast) must be due to dissociation. Faltis & Frauendorfer (47) advanced formula XXXVIII for dauricine and the matter is still under discussion.

Unquestionably the cryoscopic method for the determination of molecular weights is a fertile source of error and the reviewer would like to take this opportunity of emphasising the value of X-ray crystallography as a *routine* method of estimation of the molecular weights of crystalline substances.



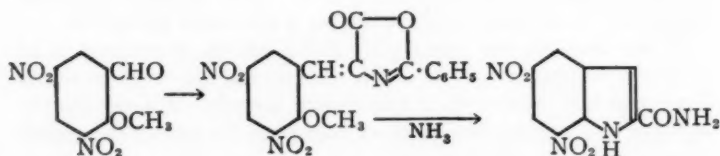
XXXVII. Dauricine (?)



XXXVIII. Dauricine (?)

*Strychnine, brucine, and vomicine.*—In this difficult field progress is continuous but nothing has emerged in the last two years which can be said to have thrown a flood of fresh light on the constitutional problem. On the other hand very useful confirmations of the correctness, essentially, of existing ideas have been obtained.

The synthesis of the amide (XXXIX) of dinitrostrychol has been effected (48) according to the following scheme, and this makes even more certain the adherence of strychnine to the indole group of the alkaloids:

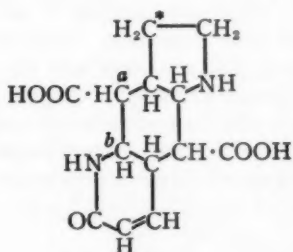


XXXIX

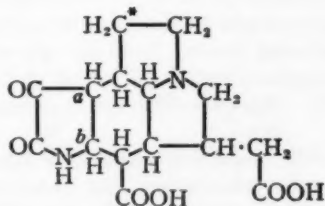
The close relation of brucine to strychnine, so close indeed that brucine may be stated to be dimethoxystrychnine, has been clearly proved in earlier work by the formation of the same products from strychnine and brucine derivatives. Hitherto, however, it has been necessary to destroy the aromatic nucleus in order to achieve this equation.

Leuchs & Overberg (49) have now accomplished the same thing by reduction and have thus offered a new and convincing proof of the identity of the stereochemical relations in brucine and strychnine. Hydrogenation of strychnidine in the presence of a very active plati-

num-oxide catalyst yields octahydrostrychnidine. Brucidine is similarly reduced in normal hydrochloric acid to the known dihydrobrucidine and then with elimination of the two methoxyl groups to the above octahydrostrychnidine. A considerable part of the activities of Leuchs and his collaborators has been occupied in applying to various brucine and strychnine derivatives the methods of oxidative degradation of the aromatic nuclei already worked out in the typical cases. For example, brucinonic acid,  $C_{23}H_{24}O_8N_2$ , the product of the oxidation of brucine by means of potassium permanganate, is further degraded by barium peroxide to an acid,  $C_{20}H_{22}O_6N_2$  ( $-CO \cdot COOH \rightarrow -COOH$ ). This latter is oxidised by 5N  $HNO_3$  to a nitroso-nitroquinone hydrate,  $C_{18}H_{16}O_{11}N_4$  (destruction of the dimethoxybenzene nucleus), and by the further action of bromine a dicarboxylic acid,  $C_{16}H_{17}O_9N_3$ , is produced (absorbs  $3H_2$  on catalytic reduction) (50). These transformations can be readily followed by means of the explanations already employed in other cases. By ringing the changes in this way on the various degradative processes and combining them in different orders a number of important compounds have been prepared. Unfortunately the question of yield becomes predominant and some of the simpler residual fragments of the strychnine molecule are accessible with difficulty in adequate amount for further study. Thus Leuchs (51) has obtained an amino acid,  $C_{13}H_{16}O_5N_2$ , from brucinonic acid by oxidation with chromic acid but the yield of this very interesting substance is extremely small (5 gm. gave 10 to 20 mg.). According to Leuchs its structure is that represented by formula XL; an acid,  $C_{15}H_{18}O_6N_2$ , obtained by the successive action of permanganate and chromic acid on the benzylidine derivatives of dihydrostrychnine or dihydrobrucine is XLI (52).



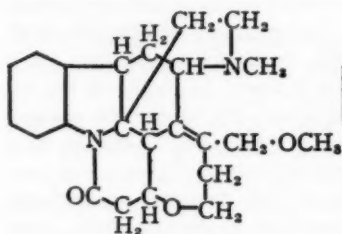
XL



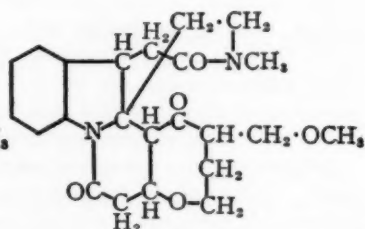
XLI

The reviewer would prefer to bring the asterisked carbon atom down to either the position *a* or *b*, but apart from this the formulae are perfectly natural deductions from the constitutions discussed in the report for 1932.

Pursuing the study of the methoxylating fission at the basic nitrogen of strychnine [N(*b*)],<sup>5</sup> methoxymethyldihydro*neo*strychnine<sup>6</sup> (XLII) is oxidised by perbenzoic acid with formation of methoxymethyl*chanodihydro*strychnone (XLIII) in good yield (53). The carbonyl group can be reduced to methylene by applying Clemmensen's method (54) and the process clearly breaks a ring which has hitherto remained intact.



XLII. Methoxymethyldihydro-*neo*strychnine



XLIII. Methoxymethyl*chanodihydro*-strychnone

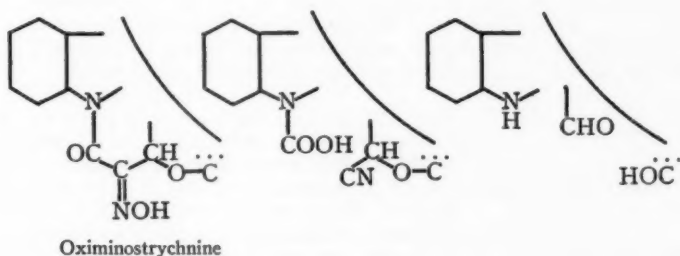
The formulae are those of the authors; Leuchs would prefer to bring the ethylene bridge into the position shown in XL and XLI.

Normal Hofmann degradations in the strychnine series are very rare but it has been found possible to prepare methines from dihydrostrychnidine (the isomeride *A* prepared by catalytic reduction of strychnidine). Thermal decomposition of methyldihydrostrychnidinium (*A*) hydrogen carbonate yields dihydrostrychnidine-*A*, two isomeric des-bases,  $C_{22}H_{28}ON_2$ , and two other substances. Catalytic hydrogenation of one of the des-bases (m.p. 197°) in acetic acid solution produces a quaternary ammonium acetate, a reversal of the usual Hofmann fission. From the new series a dihydrostrychnidine-*D* can be obtained by loss of methyl chloride from the quaternary chloride (55). This means that the strychnine ring system has been broken and

<sup>5</sup> Cf. *Ann. Rev. Biochem.*, 2, 446 (1933).

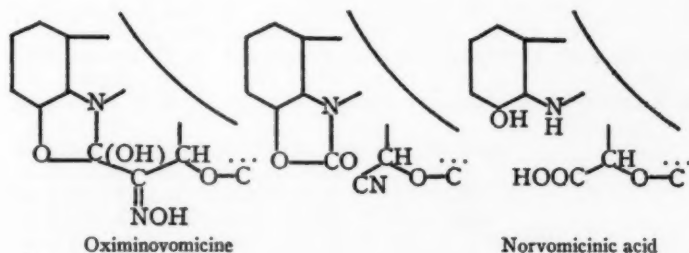
<sup>6</sup> Methylstrychninium salts treated with sodium methoxide yield this substance by metholysis at N(*b*) and wandering of the ethylene linkage into the *neo*-position.

reconstituted in a different way. Confirmation as to the position adopted for the ether oxygen in the strychnine molecule is available as the result of elegant researches of Wieland and his co-workers (56). Oximinostrychnine hydrochloride reacts with thionyl chloride with formation of a carbamic acid,  $C_{21}H_{21}O_3N_3$ , and an amine derived by decarboxylation of this. On hydrolysis by means of aqueous barium hydroxide the carbamic acid affords carbon dioxide, a cyanide, and an aldehyde-base,  $C_{19}H_{22}O_2N_2$ . These changes are represented by the part formulae below:



Curiously enough, oximinostrychnine itself is changed by thionyl chloride into the amide of the carbamic acid (some of the latter being also produced) and on hydrolysis of this carbamide (normal sodium hydroxide) a lower homologue of strychnic acid, termed norstrychnic acid, is obtained.

Oximinovomicine has been prepared (57) and this is converted by thionyl chloride into a carbamic acid lactone hydrolysed by 2N NaOH to norvomicinic acid as illustrated below in part formulae.



The study of vomicine and vomidine has been carried on in other directions but the site of the fourth oxygen atom has not yet been located. The first three oxygen atoms are those of the aromatic nucleus, the acid amide group  $[N(a)CO]$ , and the cyclic ether group.

The chief topic left for future consideration is the chemistry of morphine and sinomenine in regard to which Lutz and Small and Goto have made outstanding contributions.



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## MINERAL NUTRITION OF PLANTS\*

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The subject under discussion involves, directly or indirectly, almost all phases of plant physiology. Some of these aspects receive treatment elsewhere in these reviews. For a recent survey of mineral nutrition reference may be made to Pirschle (58). This account deals only with certain salient problems. Recent contributions to knowledge concerning the nature and rôle of the essential elements will be dealt with first, this to be followed by a discussion of present views regarding the mechanism of salt absorption in plants, which still remains an outstanding problem.

### THE ESSENTIAL ELEMENTS

*Boron.*—It is unnecessary to dwell upon the theoretical and economic importance of those essential elements required by plants only in minute amounts. Of these boron retains its interest. Its necessity is now so firmly established that a case where it was not required by a flowering plant might occasion more surprise than repeated demonstrations to the contrary. Martin (45), using water cultures, has described the deficiency symptoms of boron in particular, as well as of the more common elements, in the sugar cane. The phloem necrosis commonly associated with boron deficiency does not appear as a primary symptom in this plant and apparently 0.22 p.p.m. suffices for normal growth. McHargue & Calfee (43) have continued their investigations on boron deficiency with respect particularly to lettuce and, in accord with others, conclude that the meristematic tissues are rapidly affected. Two further papers by Warington (87, 88) deal with the relation between boron and other factors. Boron deficiency can be demonstrated at various "lengths of day," though with respect to legumes and cereals it is more acute under long than short day conditions. Boron does not affect flower production any more than the development of other meristems though absence of boron may minimise the contrasts between long and short day plants. Warington (88) also believes that boron deficiency retards calcium uptake more than

\* Received February 7, 1935.

other nutrients and this despite the difficulty that, when lack of boron can be demonstrated, absorption of elements other than calcium is already declining. According to Hoagland & Snyder (34) the strawberry plant is well adapted to the water-culture technique by which the mineral deficiencies can best be investigated. Effects due to lack of boron have been described, and apparently there is a possibility that this also may occur under certain field conditions. The incidental observation was made that the strawberry requires more boron in summer than in spring [cf. Warington (87)].

The explanation of the rôle of boron in metabolism and growth is, however, as obscure as ever.

*Zinc.*—Increasing interest attaches to the element zinc. Either as a so-called stimulant, e.g., to micro-organisms, or to alleviate actual deficiency symptoms in flowering plants, zinc has long attracted attention [see previous reviews (29, 30), also Steinberg (73), Tamiya (81), etc.]. It is, however, the recent contributions of Chandler, Hoagland & Hibbard (14) which demand discussion of this element. The observations in question originated when it was realised that the effective curative agent in heavy doses of ferrous sulphate, applied for "little leaf" of fruit trees, was zinc, present as an impurity. There is now a strong presumption that zinc is a definite requisite for normal metabolism and that the primary cause of "little leaf" is lack of this element. Nevertheless, the authors point to certain complications (in an explanation based on the assumption of a simple nutrient deficiency in the soil) and they prefer to reserve their final judgment. Other workers (cited in the work referred to) have also observed beneficial effects from field applications of zinc. Dufrénoy & Reed (21) have utilised the suggestion in the work of Chandler, Hoagland & Hibbard upon "little leaf" and are now assured that zinc deficiency is the specific cause of "mottle leaf" of citrus. These authors rightly stress the necessity for direct information of the effect of these elements on cells and believe this is conveyed by cytological observations on the cytoplasmic inclusions (62), a method of approach to nutritional problems of citrus previously used by Dufrénoy (20). These methods have been supplemented by a technique for detecting zinc in tissues which the authors believe to be specific [Reed & Dufrénoy (63)]. The emphasis upon the cell should be fostered, but the conclusion that zinc regulates oxidation-reduction potentials, which determine the reduction of nitrates, demands further evidence of the chemical mechanism by which this could be effected.

*Manganese, copper, and iron.*—Further contributions to the physiological importance of manganese, the necessity for which in certain cases is now recognised (29, 30), have been made by Saeger (67), Clark (15), Hopkins (36), and Olsen (50). Following McHargue & Calfee, whose work was reviewed earlier (30), most of these investigators utilise *Lemna*. Clark claims that the dilution of manganese which suffices for *Lemna major* is the almost incredible one of 1:3,000,000,000.

Bennett & Oserkowsky (3), actuated by the idea that there may be a connection between the physiological effects of copper and iron, traced the seasonal fluctuations of both elements in the tracheal sap of deciduous trees. Oserkowsky (51) believes that the "active," as distinct from the "total" iron in tissues, may be determined by a method involving extraction with hydrochloric acid.

During the past few years the element selenium has aroused great interest in the United States. It is a natural constituent of the soil in certain areas, where the vegetation is toxic to animals. Some species of plants may absorb this element in large amounts. Hurd-Karrer (37) has reviewed the literature pertaining to this element and has reported numerous soil- and water-culture experiments.

*Supplementary nutrient solutions.*—There is every reason to suppose that the list of essential elements is not yet complete. In water-culture technique, where good growth is the first requisite, the use of dilute, supplementary solutions finds increasing favor. These contain minute amounts of many elements which have not hitherto been regarded as essential. The term "A-Z solutions" for these is likely to persist.<sup>1</sup> The composition of two of these solutions is given by Hoagland & Snyder (34). These authors noted that a solution containing twenty-six supplementary elements produced more healthy strawberry plants than one containing twelve. An incidental observation that these differences were associated with greater resistance to mildew and red spider raises questions of considerable economic interest with regard to the rarer mineral elements. Schropp & Scharer (70) have tested the effect of Hoagland's A-Z solution (twelve elements) upon the growth of many common plants. Large increments, even exceeding 100 per cent in certain cases (sugar, beet, tobacco, maize, etc.), were obtained. Only in a few instances (rye,

<sup>1</sup> This term was proposed sometime ago by Dr. A. R. C. Haas of the Citrus Experiment Station, Riverside, Calif.

lucerne, red clover) was slightly reduced growth recorded. For many physiological purposes it is necessary to ensure that the sand- or water-culture technique does not impart unknown limitations upon the growth or response of experimental plants. The use of these or similar A-Z solutions provides some protection against this and might profitably be more widespread. The effects upon growth of cereals and legumes of a range of concentrations of nickel and cobalt [Scharer & Schropp (69)], rubidium and palladium [Brenchley (7)] have been determined. These are toxic in concentrations of the order of 0.1 milliequivalents per litre with the exception of rubidium which, though it may compete with potassium absorption [Blanck (6)] or replace potassium already absorbed [compare Brooks (12)], is apparently not injurious. Effects of various concentrations of iodide upon the growth and respiration of the tomato plant have been investigated by Wynd (91).

*The common mineral nutrients.*—Despite the increased interest in the rarer nutrients the investigation of the more familiar ones does not decline. A sulphur deficiency of tea, associated with characteristic symptoms which could be duplicated in water-culture, has been described by Storey & Leach (80) from Nyassaland, and Martin (44) has described the effects of the lack of the common nutrients upon sugar cane. Water- and sand-culture methods are being increasingly applied to fruit trees. The present position of this difficult but important aspect of mineral nutrition may be ascertained from a review by Wallace (86), and a recent paper by Colby (17). However, the most interesting developments with respect to these elements concern rather their mutual relations (particularly potassium and nitrogen) and the inter-relations between environmental factors (e.g., light) and nutrient requirement. Also the relative efficiency of ammonium salts and nitrates as nitrogen sources, particularly as it is affected by pH, has received further attention.

*Absorption of nitrogen from nitrates or ammonium salts.*—Despite recent papers the position outlined earlier in these reviews (29, 30) remains virtually unchanged. It seems to be the general experience that for optimum nitrogen absorption from ammonium salts a more alkaline reaction is required than for nitrates. The optima of pH 6.0 for ammonium salts and 4.5 for nitrates suggested by Tiedjens (83) have been, in general, confirmed by subsequent workers [Nightingale (49), Clark & Shive (16), Davidson & Shive (19)]. Tidmore (82) rightly emphasises that these experiments must not imply that

ammonium salts are invariably not toxic since this becomes very evident when the phosphate concentration is reduced from 600 p.p.m. to 75 p.p.m. It is clear that the essential reaction is not that of the solution as a whole but that at the absorbing surface. Aëration, flowing cultures, and mechanical stirring all assist in the maintenance of uniformity and have been utilised to some extent by Nightingale (49). It seems, however, that in the light of recent work upon the effects of aëration upon absorption and respiration that the whole question of the effects of external pH upon absorption should be reviewed. In experiments upon roots [Hoagland & Broyer (32, 33)] and upon storage tissues [Steward (77)], utilising rapid aëration and stirring, there seem to be grounds for questioning the old generalisation that an acid reaction per se promotes the absorption of anions. Effects of acidity and alkalinity inevitably involve also the bicarbonate ion and undissociated carbon dioxide and, unless especially prevented, these may accumulate excessively. From the effects of pH upon anion absorption in rapidly aërated buffer solutions, and of the addition of bicarbonate and carbon dioxide at constant pH, there is now considerable unpublished evidence which suggests that the oft-quoted retardation of anion absorption at high pH may not be an inevitable consequence. Returning to the discussion of ammonium versus nitrate nitrogen it seems that the conditions for maximum absorption also lead to greater utilisation [Nightingale (49), Clark & Shive (16)]. The important point here is that greater absorption from ammonium salts produces more of the soluble nitrogen fractions (asparagine, amino acid, etc.), and causes greater depletion of carbohydrate reserves. The latter effect is regarded as the chief cause of the reaction of clover to ammonium sulphate [Blackman (5)]. Pardo (55) points out that on the basis of dry weight per unit of absorbed nitrogen nitrates are still the most effective source. It is of interest here to enquire whether the increased respiration which can be obtained with nitrates<sup>2</sup> may promote the absorption of other ions and this, with the known ability of nitrate as a mobile anion to promote cation absorption, might produce, indirectly, effects upon growth which ammonium salts cannot duplicate. The little evidence available seems to indicate that the rapid penetration of ammonium tends to retard the accumulation of other ions<sup>3</sup> [see note by Steward (77)]. An extensive

<sup>2</sup> Observed both with roots and storage tissues.

<sup>3</sup> For other recent papers involving ammonium salts see Asprey (1, 2) and Homés (35).



study of the relative absorption of ammonium and nitrate nitrogen from ammonium nitrate by etiolated seedlings [Pranischnikow (61)] can only receive incidental mention. Effects of age, concentration, and external pH are described. This work is of interest because it stresses the relation to internal carbohydrate content. In young high-carbohydrate seedlings the absorption of ammonium exceeds that of nitrate but with prolonged "starvation" this may be reversed and the familiar "physiological acidity" of this salt may give place to an apparent "physiological alkalinity." Age, increased concentration, and an acid reaction all tend to increase nitrate absorption relative to ammonium. In extreme cases with old seedlings in which the synthetic mechanism is impaired, nitrate reduction may still continue and ammonium may be actually released to the external solution. These results emphasise anew that the relative absorption of anion and cation is a property not merely of the ions but is also determined by the status of the tissue.

*Potassium and nitrogen in relation to light, etc.*—The difficult problems presented by the rôle of potassium in plants have been discussed extensively in earlier reviews. Recent detailed comparisons of the biochemical characteristics of plants grown with varying potassium nutrition [Hart (27); Cattle (13)] merely suggest that, directly or indirectly, potassium affects almost all the activities of the plant. The renewed suggestion [Tincker & Darbishire (84) following earlier work of Maskell (46)] that potassium may be necessary for translocation because, under certain light conditions conducive to rapid tuber formation, potassium may limit the accumulation of dry matter in storage organs, is not wholly convincing. Any effect upon translocation is more probably imposed by the growth of the tubers and, therefore, only indirectly related to potassium nutrition.

A more hopeful approach seems to be that concerned with the mutual relationships of mineral nutrients and their inter-relations with environmental conditions. Certain of the latter have been referred to, e.g., length of day in relation to boron and potassium nutrition; also Colby (17) suggests the inter-action of light or temperature upon potassium and nitrate.

The most significant recent work, however, is that of Gassner & Goeze (23) which is germane to that of Gregory and his associates (see previous reviews; also 47, 72). One is struck by the differences of technique and this is the chief difficulty in making comparisons. Where light is a factor, as in all these more detailed nutritional investigations, its control becomes a matter of first importance. Gassner &

Goeze describe at length their technique for growing plants (cereals) under controlled conditions. Though not as elaborate as that of the California workers in its present form or of the workers at the Smithsonian Institution this has yielded results of interest. Gassner & Goeze lightly dismiss previous workers (Briggs, Gregory, Lundegårdh, etc.), asserting that they inadequately appreciated the inter-relations of the various factors. They assess the influence of nutrition and environment by measurements of assimilation, transpiration, and chlorophyll content made under standard conditions upon the first leaves of barley plants detached when ten to eleven days old. The difference between this and Gregory's technique will be noted. Gregory and his associates employ large numbers of sand cultures, in the open. The plants are grown to maturity and the physiological effects of nutrition are based not upon leaves arbitrarily selected as to age and position on the axis but upon successive leaves when it is claimed they have just reached maturity (complete expansion). Richards (64) has re-emphasised the importance of the sampling procedure. One may presume that Gregory and his school will not approve Gassner & Goeze's use of the first leaves at so early a developmental stage. Certainly of all the leaves these, being already formed in the embryo, are the least likely to be affected by the nutritional treatment. However, the differences which were obtained, and especially where these are related to light, demand serious attention. The methods of Gregory inevitably preclude light and temperature control and necessitate elaborate statistical analysis to segregate the various complicated factors. At this juncture matters of technique claim as much attention as new results. One can recognise the difficulties inherent in providing a light source which duplicates sunlight. Tottingham *et al.* (85) have described the stimulating effects upon nitrate absorption when the Mazda lamps are supplemented by short blue and long ultraviolet radiation. It seems that, despite the evident difficulties, wherever possible controlled experiments should be used (and for these the cereals are well adapted), if not exclusively, at least to supplement large-scale experiments where the casual diurnal and seasonal fluctuations of light and temperature are involved in unknown degree.

Gassner & Goeze's results may be briefly summarised as follows:

When light duration is not limiting (ten hours or more daily) a direct relation between nitrogen supply and assimilation (as well as transpiration and chlorophyll content) can be demonstrated. The connection here is that increased protein determines the number of chlo-

roplasts. With short days (three hours per day) light is limiting and no response to nitrogen is revealed. With normal light duration (twelve hours) the effects of increased nitrogen supply increase with age (twenty-five days compared with ten to eleven days).

The essential point with respect to potassium nutrition is that its effect depends also upon nitrogen and must be investigated under conditions of age, light, and nitrogen, where none of these factors is limiting. It was found that under these conditions the highest assimilation rates (uncorrected for respiration) were obtained with the lowest potassium supply. In this (23) and a previous paper (22) evidence is given of a low but optimum potassium supply below which assimilation decreases rapidly and above which it declines gradually to the highest potassium dosage. Apparently the potassium effect, unlike the nitrogen response, may be demonstrated under short day conditions. Gassner & Goeze stress the fact that potassium and nitrogen must be considered together and they believe that it is their respective effects upon protein synthesis which are most fundamental. High potassium and low nitrogen produce similar results upon protein content, chlorophyll content, and assimilation and transpiration rates and it is the factor present in "relative excess" which predominates. The high assimilation of young leaves with comparative potassium deficiency may be, therefore, regarded as also due to "relative nitrogen excess." Only at extreme potassium deficiency would these harmonise with other views of the relation between potassium and assimilation.

The present status of the views of Gregory and his associates upon the specific effects of potassium and nitrogen upon the metabolism of the barley plant may be summarised for comparison with the above. Here only casual reference can be made to two manuscripts [Sen (72) and Mathur (47)] which contain very detailed data concerning the effects of potassium and nitrogen nutrition upon carbohydrate and nitrogen metabolism as well as the respiration and growth of barley plants. It will be recalled that previous papers have stressed the fact that nitrogen deficiency results in high sugar content and decreased total nitrogen and amino nitrogen, whereas potassium deficiency produces a lower sugar level in leaves and increased amino acid nitrogen and amide nitrogen. A low level of potassium nutrition which would then place nitrogen in "relative excess," to return to Gassner & Goeze's view, was here associated not with high assimilation (not measured) but with high respiration. This obtained unless the level of potassium nutrition was so low that the sugar content really limited respiration.

The two series of investigations are at least similar in that, with respect to two processes, optima of physiological activity are determined by potassium nutrition and in both cases the effect involves nitrogen metabolism. In the one (assimilation) protein nitrogen seems to be the determining component and in the other (respiration) amino acid and amide are more intimately concerned. Space prohibits further discussion of the many interesting, and not always concordant, aspects of these two important series of investigations.

*The absorption and accumulation of mineral nutrients.*<sup>4</sup>—The extensive literature now available (for recent summaries see 52, 53), to which notable additions have been made in recent years, testifies to the interest in this question. However, it is becoming increasingly more difficult for the general reader to isolate from the investigations and conclusions of different schools and workers those general principles which may be applied to the problem of nutrition. The diversity of experimental material and technique now being employed threatens to obscure rather than elucidate. The organisms selected are usually chosen arbitrarily to simplify some particular phase of experimental approach. It is, however, evident that their morphological peculiarities, state of development and previous nutrition, and their response to further growth and differentiation impart limitations upon salt accumulation, the significance of which has not always been adequately appreciated. The time seems opportune for a review of the most outstanding investigations from this point of view.

It is now evident that, quite apart from the strictly nutritional aspects, the absorption of mineral elements is determined by, and related to, the capacity of cells for further growth and metabolism [Berry & Steward (4) and previous papers of this series; also see 29, 30]. This relation between salt accumulation and growth must of necessity be complex. It is common to regard salt absorption as an essential prerequisite of growth, but, as the later argument will show, there is much to be said for the reciprocal relationship. The obvious factor that during cell extension vacuoles increase in size and thus provide an internal phase for dissolved solute is not the only one. Growth (extension and cell division) also involves the utilisation of energy released during active metabolism, and it is probably here that the fundamental relation to salt accumulation lies. A cursory glance at the various systems which have figured prominently in salt accumu-

<sup>4</sup> Cf. also this volume, p. 5. (EDITOR.)

lation studies<sup>5</sup> reveals the fact that these display a wide variation in their capacity for active growth and rapid metabolism. It is the extent to which this capacity determines the ability to accumulate salt which must be discussed first.

Figure 1 reveals the general relationship between these various systems from the point of view outlined above. These qualitative comparisons<sup>6</sup> are based largely upon quantitative experiments under controlled environmental conditions identical with, or approximating to, those outlined for storage tissues (74) and utilising potassium bromide as the absorbed solute. For the purpose in view a readily absorbed, non-metabolised salt is essential.

The meristematic cell is the logical starting point for any survey upon the relation of growth to active salt accumulation. For strictly meristematic cells there is, unfortunately, little or no precise evidence. By a pardonable extension of the results obtained with other systems (e.g., storage tissues and roots) there is a strong presumption that the intensity of salt accumulation measured by the concentrations obtainable would be at a maximum. Investigation upon the distribution of mineral nutrients (especially potassium, the most readily accumulated cation) indicate their relative abundance in the more active tissues.<sup>7</sup> As emphasised in Figure 1, however, almost all experimental investigations which have been concerned specifically with the accumulation mechanism have utilised cells which during differentiation have departed in varying degrees from the condition of active cell division.

The work of Hoagland & Broyer (31, 32) with young developing

<sup>5</sup> Species of green algae, both marine and fresh water, attached and detached root systems of higher plants, cut portions of dormant storage organs (both modified shoots and roots), epidermal tissues, etc.

<sup>6</sup> It is not possible to make this survey entirely by reference to published results. The writer has, however, drawn in part upon much unpublished data obtained in the Department of Botany of the University of Leeds, the Carnegie Laboratory at the Dry Tortugas, and the Division of Plant Nutrition of the University of California. Most of the relevant facts were presented at the symposium on salt accumulation at the meeting of the American Association for the Advancement of Science at Berkeley, California, in June 1934.

<sup>7</sup> James & Penston (40) remark that: "There is thus a close connexion between abundance of potassium and active growth, but there is no evidence that the potassium precedes and provokes the growth. The alternative that the growing tissues have the capacity for collecting the potassium that they may require for further growth is equally allowable and on the evidence of other lines of work more probable." (See also earlier papers of this series.)

## SALT ACCUMULATION RELATIVE TO GROWTH AND METABOLISM

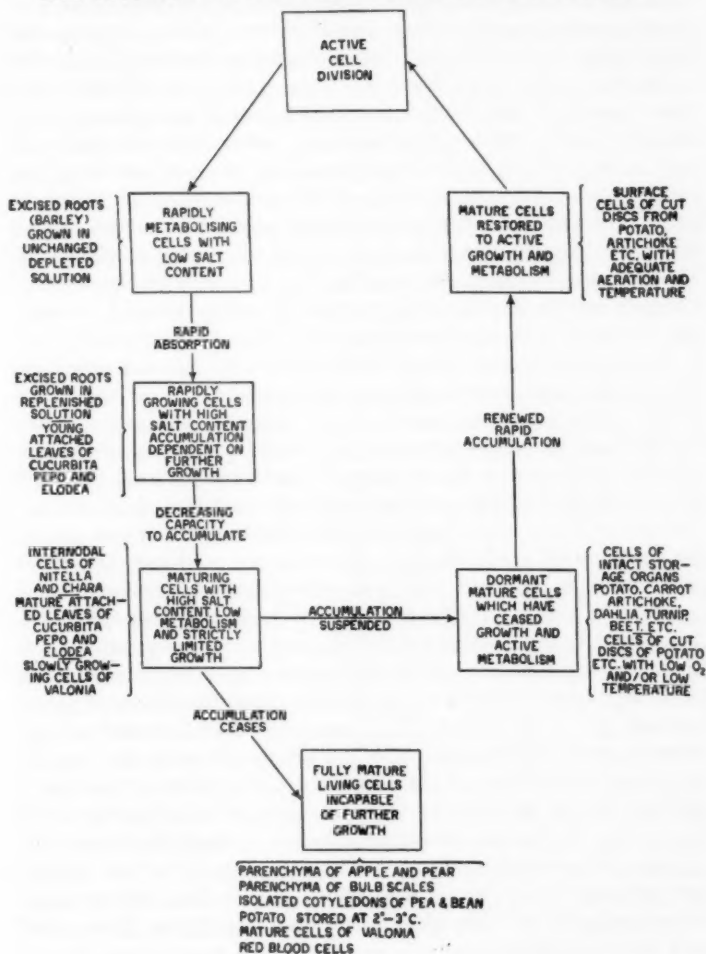


Fig. 1.—Classification of representative types of plant tissues and cells with regard to their ability to accumulate inorganic solutes.

roots of cereals shows clearly the relation of salt accumulation to previous nutrition and development. When grown in a limited supply of dilute nutrients, seedling plants can be obtained rich in carbohydrate but in which the vegetative growth is limited by the supply of readily accumulated salts (e.g., nitrates). Root systems, so obtained, have been depleted of salts by the more active cells of the growing shoot and if isolated from it will absorb very rapidly large amounts of a readily accumulated solute (e.g., potassium bromide) to very high concentrations. (A concentration of the order of 100 milliequivalents per litre may be attained in ten hours from an external solution of 7.5 milliequivalents per litre.) As in the case of the storage tissues (4, 74, 75, etc.) so with these root systems, the accumulation demands an adequate temperature and oxygen supply as well as cells rich in sugar, much of which is metabolised during the process. An interesting point is that the root systems which develop under the low-salt conditions attain higher sugar concentrations than those in a full nutrient. Both factors, namely, high-sugar and low-salt content, combine to produce high-salt absorption when the supply is available. Clearly one is dealing here with a system having a great potentiality for further growth and capable of maintaining an active rate of metabolism (e.g., respiration and oxidation of carbohydrate) but whose initial low-salt content has been limited, not by the capacity of the cell to absorb, but of the environment to supply. Under these circumstances the cells in young root systems absorb up to the limit of their present capacity, so rapidly that growth in the sense of cell division is not a primary factor. It is clear, however, that this salt-absorption has been deferred and by this means segregated in time from the growth and differentiation it would normally have accompanied. In this category of cells, formed and developed under minimal salt supply, but which still retain the capacity for active metabolism, are the systems which exhibit, at least for a brief period, the most intense salt-absorption of which the writer is aware. Any further accumulation, however, is dependent upon and occurs simultaneously with the continued development of the constituent cells and the system is then subject to the same limitations as if the developing cells had accumulated up to capacity at all stages of their previous development. In any work upon root systems it is a matter of importance to stipulate and evaluate these factors, with the added difficulty that for attached roots rapid translocation may simulate the condition produced by low external supply.

It is well to emphasise here that an individual root system is not a



homogeneous unit. The investigation of Prevot & Steward (60), upon single unbranched primary roots, has established the view that when they are grown with full salt supply the most active accumulating region is that, including the apex itself, which consists of meristematic as well as elongating and occasionally dividing cells. A steep gradient along the root axis terminates in mature cells whose minimal activity may be ascribed alike to the cessation of growth and adequate previous supply. When roots are grown under low-salt conditions these gradients due to growth may either persist or be masked by (a) the much enhanced absorption of the root as a whole, or (b) the fact that excessive deficiency may impair the efficiency of the apical region.

It is convenient to interrupt the main argument to remark that the use of quantitative technique reopens the problem of the absorbing surface of roots. The problem clearly involves (a) penetration, (b) accumulation. In so far as they were able to discuss solutes Scott & Priestley (71) in the most coherent account of the root system<sup>8</sup> had the former chiefly in mind. The new results of Hoagland, Prevot, etc., while they do not detract from the interest of the endodermis, stress rather the function of the cortical cells, which appear, at least for some weeks, able to reabsorb salts after they have been depleted by the growing shoot. The extent to which they contribute to the functional absorbing system is, therefore, determined by the balance between the demands of the shoot and the external supply. In the opinion of the writer some of the outstanding problems of nutrition refer to the behavior of the root. Of these the need for an adequate elucidation of the metabolic basis for the observed gradients of accumulation along a primary root is evident. The contributions of Lund and his associates [see Henderson (28)] are of interest in this connection.<sup>9</sup> Secondly, the mechanism whereby cells, whether cortical or endodermal, release salts to the stele is fundamental and still obscure. In the light of recent work the oxygen relations of the endodermis may supplement the emphasis which Scott & Priestley placed upon carbon dioxide and pH in this regard. That a given solute may be concentrated in the tracheal sap is now beyond all question, as shown by experiments on plants growing in soil by Pierre & Pohlmann (57)

<sup>8</sup> Reference may also be made to Popesco (59). Crider's observations (18) do not necessarily mean that the woody parts of roots contribute to the functional absorbing surface.

<sup>9</sup> The tissue-culture technique of White (89, 90) for root tips should also be noted.

and Russian workers [Sabinin (66), Schardakoff (68)].<sup>10</sup> Much of the evidence at present available, however, indicates that this secretion into the stele requires metabolic activity similar to that of the absorption itself and is determined by similar variables (31).

Except under the special circumstances outlined above, salt accumulation will be limited by the capacity of cells or organs for further growth. Figure 1 illustrates the fact that cells or organs which have progressed far in their development exhibit declining accumulation. This can be readily illustrated by the basipetal gradients of accumulation in leaves borne upon a single unbranched stem. Table I refers to data obtained with vegetable marrow plants<sup>11</sup> grown in a large volume of nutrient, maintained complete. The leaves were sampled after a brief period of contact with nutrient containing dilute potassium bromide. Clearly on a given stem the leaves most actively growing attain the greatest concentrations.

TABLE I\*

Leaf Number (Ascending Order of Age)	Mg. Bromine per gm. Dry Weight		
	Case 1	Case 2	Case 3
1.....	5.18	2.16	3.53
2.....	5.14	4.87	3.51
3.....	9.02	8.01	6.72
4.....	12.22	8.84	6.61
5.....	10.02	9.46	5.57

\* Data of A. G. and F. C. Steward.

It is the general relationship to growth with which we are here concerned. Clearly the complete elucidation of these phenomena demands a knowledge of the complete metabolic behavior of successive leaves on the same axis at a given time. Though this is not available, the data of Cattle (13), and of Gregory & Sen (72) are highly suggestive that significant parallelisms would be revealed. Here again it is important to recall the factor of previous nutrition. Similar experiments with another variety of *Cucurbita pepo*<sup>12</sup> grown in large tanks

<sup>10</sup> This is in agreement with unpublished results of water-culture experiments by Steward and by Hoagland.

<sup>11</sup> *Cucurbita pepo* (vegetable marrow, var. Sutton's Long Green); plants about 3 ft. long.

<sup>12</sup> *Cucurbita pepo* (Hubbard squash, var.); capable of much more vigorous vegetative growth than vegetable marrow; forms very large leaves and plants up to 20 to 30 ft. long.

under California conditions, conducive to very vigorous vegetative growth, show that instead of salt accumulation being limited by growth, the latter outstrips the former, so much so that even cells of foliage leaves, a great distance behind the apex, can still accommodate considerable salt if they can receive it and, in fact, do so when the plants are transferred to potassium bromide solutions. They behave, in short, to some extent like the "low-salt roots" previously referred to. Under these conditions, the basipetal gradients of salt absorption determined by growth may be obscured, at any rate until the deficit of total salt is obliterated.

Confirmation of the existence of basipetal gradients of accumulation is also available from Rosenfels (65) who worked with *Elodea* previously grown in pond water and transferred to potassium bromide.

The large, multinucleate cells of *Valonia*, *Chara*, and *Nitella* species, in view of their wide use, demand special attention. They are clearly much less capable of further growth than the preceding examples and, having been bathed by a solution rich in salt, they are initially, as their actual concentrations show, of high salt content. Even with these cells there is evidence that the younger have greater concentrations, which is not in agreement with statements that the sap composition remains constant during growth. Results of a carefully controlled series of collections of *Valonia ventricosa* are given in Table II.<sup>13</sup>

TABLE II

SAP CONTENT OF *Valonia ventricosa* IN RELATION TO CELL SIZE

Wt. per cell, gm.*.....	0.43	0.86	1.50	2.88	4.6	7.9
Chloride in equivalents per litre...	0.632	0.628	0.624	0.620	0.611	0.609
Vol. per cell, cc.†.....	0.70		2.50	9.0		18.0
Chloride in equivalents per litre.....	0.637		0.627	0.617		0.611
Potassium in equivalents per litre.....	0.627		0.613	0.611		0.601

\* Steward (76).

† Steward & Martin (79).

If cell size may be safely regarded as an indication of maturity, the declining accumulation illustrated in Figure 1 seems evident. Their strictly limited growth, high initial salt concentration, and extremely low rate of metabolism adequately account for the relatively slight salt

<sup>13</sup> See also incidental reference to cell size in works of Osterhout and others (38, 53).

accumulation even under conditions in which more active cells (e.g., of roots or storage tissues) would rapidly absorb (76). Indeed it is quite evident from the earlier work on *Nitella* that the duration of time and the conditions of accumulation did necessitate further growth. *Valonia* species as commonly investigated, removed from the normal habitat, show even less capacity for growth than a miscellaneous population of *Nitella* cells and a metabolic rate which, when compared with roots or storage tissue, seems negligible. The writer has made such direct comparisons with similar technique using *Valonia* cells and concluded that, under conditions of bromide concentration, temperature, and aëration which would induce both rapid respiration and active salt accumulation in either roots or storage tissues, both these activities were almost negligible in *Valonia*. Not only the detailed manner of growth but also the metabolism of *Valonia* species is largely conjecture, while the variables which determine its normal behavior in a highly restricted and specialised habitat are quite obscure. The extremely sluggish behavior of these cells appears to offset their apparent morphological advantages as a basis for a general theory. Furthermore, until the manner of growth and general metabolism of *Valonia* is more clearly defined, it must be regarded rather as an anomalous than a typical case.

These relatively mature, large cells, therefore, are to be regarded at best as near the limit of their vegetative activity and even in many cases completely incapable of further active growth. In the mind of the writer such systems find their parallel rather with those mature storage parenchyma cells which have lost the capacity for renewed growth and which Berry & Steward (4) find virtually incapable of salt accumulation even under apparently favorable environmental conditions. It seems that the ability for metabolism, growth, and salt accumulation of such cells<sup>14</sup> is really reminiscent of that exhibited by red blood corpuscles which, being devoid of further capacity to grow, are restricted in their salt relations to exchanges involving ions absorbed during previous differentiation when the cell retained greater activity.

It is quite evident that the most active systems are characterised by the ability to absorb both anion and cation simultaneously. The young developing root systems take in both potassium and bromide in approximately equivalent amount. As the tendency for growth decreases

<sup>14</sup> See note by Steward (77) for brief survey of recent work.

and the total salt absorbed becomes less, ion exchange becomes an increasingly conspicuous property of the cells. *Nitella* is an intermediate case, since it will be recalled that, of the total bromide absorbed, some is exchanged for chloride but the remainder is accompanied by potassium. It is clear that ionic exchange presupposes some previous salt absorption and an explanation of exchange alone, however complete, could not suffice for the more active systems with which we are primarily concerned.<sup>15</sup>

<sup>15</sup> Undesirable as it is to multiply terms these two processes should be segregated. The existing terms "salt accumulation" and "ion exchange," have been so variously used that their restriction would cause confusion and the second has been regarded as an essential step in the former. It seems that the term "primary salt absorption" could accurately describe that which occurs in those actively growing, metabolising systems which absorb in the vacuole both anion and cation, gain rapidly in total salt and often also in osmotic concentration. Clearly, this is the major problem. For those cases where relatively mature cells, by virtue of a change in their external environment, re-adjust their salt content without gain or loss of concentration of dissolved salts, the term "induced absorption" would appear to be suitable. These considerations seem to have been appreciated by Briggs (8, 9) who, however, seems to misplace the emphasis upon "accumulation in the sap" and "ionic interchange" in some of the cases he discusses. Even so the latter category would still include dissimilar processes. On the one hand there are those well-known cases where a given ion replaces another, initially in the vacuole as, e.g., bromide for chloride and in which the metabolic activity of the cells participates. These must be distinguished from mere reactions or base exchanges concerned primarily with the constituents of wall or protoplasm (24, 25) or rapid penetration of a base like  $\text{NH}_4\text{OH}$  which can rapidly displace other cations. Examples of the latter include many apparent cases where absorption by cells (usually of dyes or cations) appears unrelated to vital processes and is determined solely by the mass law, adsorption isotherm, or other simple relation. The writer suspects that the work of Asprey (1, 2), in strong unaerated salt solutions during short periods, is concerned chiefly with such secondary or "induced effects" and not with "primary absorption" in the vacuole except in minimal degree. Briggs (9) has added to his speculative writings a further contribution which develops extensively the case of storage tissues entirely on the basis of "ionic exchange." It is regrettable that this treatment is based upon arbitrary assumptions (see p. 306 and compare 78, p. 117) which are clearly untenable and with a totally mistaken view of the capacity of the tissues concerned for further growth and metabolism. Even so it is difficult to appreciate the value of the attempt to elaborate the theory by "hypothetical data." These considerations have been set forth at length because there seems to be real danger that the major problem becomes obscured especially by the failure to realise when certain systems, by virtue of their past history or present environment, are inevitably limited in their capacity for the type of salt absorption designated "primary."

Clearly, however, an irreversible decrease of the capacity for salt accumulation in development is not characteristic of all plant cells. In many cases this appears to be the inevitable sequence and culminates in the almost complete absence of "primary absorption." On the other hand, parenchyma cells, which, for a variety of reasons, pass into a condition of reduced metabolic activity, during which for long periods of time accumulation of salts is suspended, may subsequently embark upon a period of renewed salt absorption provided the circumstances permit also the requisite rapid metabolism. This behavior is illustrated by a variety of massive storage organs [Berry & Steward (4), and see Figure 1].

The significant feature is that conspicuous accumulation of the type designated "primary absorption" seems to be inevitably associated with cells which retain the capacity to divide. Cells which have irreversibly lost this capacity cannot be induced to recommence active accumulation despite the most favorable conditions of aëration, etc. (4). On the contrary they may not even retain their existing salt content. The latter condition is not to be confused with death. Such cells may retain their turgor and respire for long periods even at a high rate but they lack some more fundamental attribute of which the capacity to grow and divide is the best indication at present available. The metabolic significance of this may be that such cells are actively engaged in protein synthesis.

An unexpected confirmation of the point of view summarised above and expressed in (4) accrues from the observation that potato parenchyma may exhibit both types of behavior according to the conditions. After prolonged storage at 36° F. the cells lack both the normal capacity to form a meristem and the ability to accumulate salts from dilute aërated solution. Both of these properties return, apparently simultaneously, when tubers are transferred from 36° F. to room temperature for a protracted period. It is significant that, without the more fundamental ability to grow and divide, even the unusually high respiration of the sugar-rich tubers will not alone suffice to bring about accumulation.

It seems to the writer particularly important that all systems investigated in salt absorption be accurately evaluated with respect to these two factors: (a) capacity for further growth and active metabolism, (b) previous nutrition. Many apparent contradictions may then disappear. Either the scheme of Figure 1 or some other formulation is necessary to classify accumulating systems in order to prevent con-

fusion between incomparable ones and to insure that generalisations are drawn only from typical cases. The most active systems in which the details of metabolism can be followed are the most profitable. It is now no longer in doubt that the accumulated salts remain in true solution and for present problems the apparent advantages of large cells are more than counterbalanced by their metabolic inactivity.

*The rôle of metabolism.*—Of the vital functions causally related to salt accumulation, respiration has received most attention. The work on storage tissues continues to show that the conditions for maximum salt absorption and respiration coincide (75, 78). An apparent exception to this general rule [Briggs & Petrie (10)] appears to be ill-founded [Steward & Berry (78)]. The effect of oxygen as a limiting factor in both processes has been demonstrated for potato (75), and for carrot and artichoke (unpublished experiments). There can no longer be any justification for neglect of this variable in any absorption experiments.<sup>10</sup> Petrie (56) has published data obtained by using whole plants for long periods (twelve days) aerated at two oxygen tensions (20 and 3 per cent). Greater respiration, growth, and salt absorption were found at the higher oxygen tension. Hoagland & Broyer have shown (32) that unless aerated with a mixture richer than 7.5 per cent oxygen, the sugar metabolism of barley roots as well as their respiration and salt accumulation (potassium, halide, nitrate) in the sap may all be limited by oxygen. The form of the curves is strikingly similar to that found for potato (75), but differs mainly in the critical oxygen tension. The same type of curve has also been obtained by Lundegårdh & Burström (42). It is of interest that potato roots grown in water resemble barley roots more closely than potato tubers in this particular. Similar limitations on the absorption by *Elodea* at low-oxygen tensions or with a lack of aëration were observed by Rosenfels (65).

While respiration has figured prominently in the theories of Osterhout and his school (52, 53, and works cited), of Brooks (11), and of

<sup>10</sup> Asprey (1, p. 670) with peculiar logic satisfies himself that a closed unaerated system suffices for comparison between salts because the conditions are described as "comparable." A following paragraph tacitly admits that the absorption behavior of the tissue was limited by aëration, but Asprey still fails to appreciate that, until this factor is standardised, no other variable can be studied effectively. At this stage, it is also regrettable that Homès (35) in his paper on the absorption of chlorides, failed to consider the present status of knowledge.



Briggs (8) concerning the accumulation in *Nitella* and *Valonia* there are no recorded comparisons between the two processes for these organisms. In the writer's opinion this is regrettable. Lundegårdh & Burström (41), however, have measured respiration and salt absorption simultaneously. One is struck with the large variations within one experiment and between replicates which are tolerated by Lundegårdh. These suggest that some of the essential factors remain uncontrolled.

The following views of the rôle of respiration have been suggested either severally or together by different workers:

1. In conjunction with an arbitrarily designed membrane system, respiration is supposed to operate by an exchange of hydrogen ion and bicarbonate ion for entering anion and cation (Brooks, Briggs, etc.).
2. Since unequal absorption of anion and cation is common, respiration may supply ions to preserve ionic balance.
3. Where a gradient of acidity between vacuole and external solution is a cardinal feature of the suggested mechanism the rate of respiration is assumed to determine the internal pH and, therefore, the magnitude of the gradient (Osterhout, *et al.*, 52, 53).
4. An unusual point of view that salt absorption regulates respiration because entering anions require an excess production of bicarbonate but that respiration is not causally related to cation absorption is favored by Lundegårdh & Burström (41).

It is the writer's view that none of these is adequate. The reasons for this drastic statement may be summarised.

There is no simple quantitative relation between respiration and salt accumulated, but rather a general parallelism [storage tissues (75, 78; also earlier papers), excised roots (32)]. Active cells produce much more carbon dioxide than the chemical equivalent of the salt absorbed and yet changes in the total respiration invariably result in differences in salt absorption. Cells limited by oxygen may still produce carbon dioxide at a rate which ought to suffice for salt absorption on some of the theories mentioned but may, on the contrary, lose ions (75). Bicarbonate ion is unnecessary to preserve ionic balance where anion and cation are simultaneously absorbed in equivalent amount. The fact is often overlooked that the vacuoles of flowering plants are stabilised against drastic changes of reaction, not only by their own buffer systems, but also by metabolic processes. The ready

response of internal reactions to changes in respiration cannot be accepted merely upon *a priori* grounds. In this respect Osterhout's model (52, 54) in which the vacuole is represented by an unbuffered solution through which carbon dioxide is bubbled represents an undue simplification. Those cases where cells, though actively producing carbon dioxide, yet fail to accumulate salts [Berry & Steward (4)], present special difficulties with respect to any view based upon the direct effects of mere carbon dioxide production. Similarly the accumulation of anions in the light by green cells during absorption of carbon dioxide [really evident in the earlier *Nitella* work but even more convincingly shown with carbon dioxide measurements by Rosenfels (62) for *Elodea*] again imply that it is some aspect of metabolism other than carbon dioxide production per se which is concerned.

It is difficult to subscribe to the view of Lundegårdh & Burström (41) that respiration is concerned only with anion and not with cation absorption in view of the clear evidence to the contrary (75, 32). Lundegårdh & Burström's view that there is a special respiration component due to salts (anions) is not supported by any data of which the writer is aware. It is agreed that nitrate and phosphate have a direct effect upon respiration which cannot be evaded, but there is excellent evidence that with non-metabolised salts their direct effects upon respiration may be small and in dilute solution negligible (75, 78). The view that respiration of roots comprises two components related by the expression  $R_t = R_g + kA$  (where  $R_t = \text{Totalatmung}$ ;  $R_g = \text{Grundatmung}$  and  $kA = \text{Anionatmung}$ ,  $k$  being a specific constant for each anion) is due to Lundegårdh & Burström (41). Apparently (42) *Grundatmung* is entirely aerobic while *Anionatmung* may be anaerobic. These workers utilise a technique in which the rate of aeration is fixed by the dimensions of the apparatus rather than by the needs of the tissue. Furthermore the absorption data clearly involve, but do not discriminate between, both shoot and root, while the respiration figures with which they are compared refer only to roots and neglect the effects of the leaves. The relation  $R_t = R_g + kA$  is derived from a large number of very variable, isolated experiments. If the result is to be of permanent value it must be certain that it is not fortuitous, and should be based upon evidence derived from parallel cultures which, except in the amount of absorption procured, are strictly comparable (as to age, previous nutrition, sugar content, absorbing surface). This does not appear to be so for the data in question. The only safe conclusion seems to be that high absorption is

usually accompanied by higher total respiration and the theory of "Anionatmung" seems unproven. Salt absorption seems to vary rather with the general level of respiration, which is determined by variables other than salt accumulation (oxygen, temperature, sugar content, etc.), rather than with a special component of it due specifically to the presence of salts. It is unnecessary to recapitulate here views upon the rôle of respiration which are described elsewhere (75, 78, 4). It seems impossible to evade entirely the function of respiration as a source of energy but it is equally clear that it is its relation to the activity of the system as a whole and not directly to salt absorption which is relevant. As emphasised elsewhere in this review high respiration does not inevitably lead to high accumulation if other properties are absent. It is evident that the biochemistry of salt accumulation is a virgin field. All actively accumulating systems are conspicuous for their rapid utilisation of carbohydrate and the ultimate details of the relation of this to salt absorption are salient problems for the future.

Further contributions of Osterhout and his associates call for separate comment. These are mainly concerned with further study of the model systems described in an earlier review (30) but also provide new data upon *Valonia* (39). This now extensive literature pertaining to *Valonia* presents the greatest difficulty to the general reader. Space prevents an adequate summary but reference may be made to Osterhout (53). There are two major questions: (a) does the theory adequately account for the behavior of *Valonia* upon which the experiments are made? (b) If so, should the principles be extended to cells in general? Of these, the second is more important than the first. The writer has discussed this matter briefly elsewhere (77). Figure 1 and the ensuing discussion will indicate that this obscure organism is not as comparable with rapidly absorbing, growing and metabolising cells as many imagine. Unfortunately, apart from the indirect bearing of the effects of light, the work upon *Valonia* provides no data upon which the metabolism of the organism may be assessed. Even the rôle of respiration in providing carbon dioxide, or other acids demanded by the theory, is assumed and not based upon actual measurements.

A close perusal of some of the more crucial experiments arouses grave doubts concerning the fundamental postulates. The views upon the nature of the membranes were inherited from the still controversial interpretations of electrical experiments but need not concern us here. However, the entrance of bases, only in the form of undisso-

ciated molecules ( $\text{KOH}$ ,  $\text{NaOH}$ ,  $\text{NH}_4\text{OH}$ , etc.), and the theory of "thermodynamic potentials of free base" is fundamental. This idea is the cardinal feature of the theory and is obviously the basis of the "model experiments." It is evident that this theory arose out of, and was mainly tested by, experiments in which the observed movements of potassium and sodium were with and not against the concentration gradients. This was mainly due to the unusual concentration relations of *Valonia* and its environment. Because this will occur with any treatment affecting the vitality of the cell, all such experiments [for example those involving ammonium chloride (38) or external pH's of 5.5] are of questionable value as a basis for a theory which must explain movements in the reverse direction (77). The most extreme case of this tendency to utilise movements of potassium and sodium, which are clearly due to injury, in confirmation of the theory is that quoted by Osterhout (53, p. 987) in which *Valonia*, which only grows under specialised conditions of light and high temperature, was placed for several days in a dark ice-box! An impartial view must discard such evidence. In fact one can clearly discern that the theory, in any general application, only obtains a semblance of validity because of two special facts, (a) the high sodium content of sea water and (b) the alkalinity of this medium. Tested by the quantitative behavior of almost any fresh-water organism or flowering plant the theory fails conspicuously, because in these systems which tolerate the same or more acid solutions than their vacuoles, a steep pH gradient between vacuole and external solution is not an essential condition for accumulation. This point has been re-emphasised on the basis of striking data with *Nitella* and root tissues by Hoagland & Broyer (32). Even for *Valonia* the crucial test of the theory would be an increased accumulation of potassium produced by increased external pH. Until recently this was lacking. A subsequent paper (39) claims to establish this, but it is plainly unconvincing. Experiments in the dark were inconclusive, and in the light slight and sometimes vague (Fig. 1, p. 732) changes of external reaction ascribed to photosynthesis produced, not increased internal concentration, but volume changes. These data are interpreted to mean that a very small pH difference in the direction of alkalinity causes a considerable change of total-salt content. In the note referred to (77) data are summarised which seem to indicate that in solutions enriched with potassium chloride the behavior of *Valonia* is determined by the concentrations of potassium and chloride but relatively independent of the hydroxyl concentration over a wide range. In

short, the theory is still doubtful as far as *Valonia* is concerned and is clearly inapplicable to many other tissues, especially where strong bases are concerned. With this in mind the guaiacol-cresol models, whatever their physicochemical interest, are too far from physiological reality to be discussed here, notwithstanding the recent elaborate treatment of these systems (54). Future progress will probably be determined by detailed studies of the metabolic and vital processes accompanying salt accumulation using the most active systems available and rigorously controlled conditions. For this *Valonia* involves unnecessary difficulties while models merely evade the real issue.

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## GROWTH SUBSTANCES IN PLANTS\*

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### HORMONES IN HIGHER PLANTS

Since the subject has not been reviewed before, the literature will not be confined to that of the past year or two only. However, the more botanical aspects of the subject have been reviewed by Stark (76) (early work), F. A. F. C. Went (93) and Snow (70) (general), Du Buy & Nuernbergk (25) and Rawitscher (57) (tropisms), and lately again by F. W. Went (99).

### CELL ELONGATION

Much of the work has been done on the cylindrical primary leaf-sheath, or coleoptile, of the grasses, usually *Avena*. In this organ all cell divisions are completed at a very early stage, and subsequent growth consists entirely of cell elongation. It was first shown by Boysen-Jensen (9) and confirmed by Páal (56) that if the tip of an etiolated coleoptile were removed, in darkness, and replaced over gelatine, then on illuminating the tip from one side phototropic curvature appeared not only in the tip, but also in the part below the cut. Stimulus was therefore conducted across the discontinuity. Páal further showed that if the tip were replaced a little to one side of the stump, a curvature appeared away from the applied tip, in darkness, i.e., the tip promoted the growth of the part below it. Careful growth measurements by Söding (72) confirmed the growth-promoting effect of the tip. It follows that a substance is secreted by the tip which migrates toward the base of the coleoptile and controls its growth, both as regards symmetrical and unsymmetrical (tropistic) growth.

Final proof of the existence of this growth substance was obtained by Went in 1928 (95). Experiments by Stark (75) and Seubert (64) had shown that the application of small blocks of agar or gelatin, containing various test preparations, to one side of decapitated coleoptiles caused curvatures away from the block if growth-promoting, and toward the block if growth-inhibiting in their action. Seubert found saliva, diastase, and malt extract to be growth-promoting. Went

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showed that if the tips from a number of coleoptiles were placed on agar, then the agar produced strong curvatures of the growth-promoting type on decapitated coleoptiles. Further, he adapted the method for quantitative assay of the activity: the curvature produced by standard agar blocks, under standard conditions of temperature and humidity, etc., was shown to be proportional to the number of tips which had stood upon them, or to the time during which a given number of tips had stood. In other words, the curvature was proportional to the amount of the growth substance which had diffused into the agar. The proportionality holds strictly up to a limiting maximum curvature, and by taking a suitable number of test plants an accuracy of 5 to 10 per cent could readily be obtained. The substance was shown to be thermo- and photo-stable and readily diffusible. To get an idea of the molecular size, Went used a diffusion method which might find a more general application on account of its simplicity. The substance was allowed to diffuse from agar into a series of blocks of fresh agar and then the activity of each block assayed. The diffusion coefficient could thus be calculated, and the corresponding molecular weight was found to be 376. The substance is therefore relatively simple.

Went showed, further, that if the substance be allowed to diffuse from the two sides of a unilaterally illuminated coleoptile into separate agar blocks, then the shaded side yielded more than the lighted side. Dolk (22) found correspondingly that after placing the coleoptile in a horizontal position the lower half yielded more growth substance than the upper, the total amount remaining the same. Hence geotropic and phototropic responses result primarily from unequal distribution of the growth-promoting hormone on the two sides of the coleoptile. That this must be the mechanism of tropisms was in fact realized earlier by Cholodny (16) who put it forward as a definite theory. Similar hormonal control exists in other suitable research objects [Beyer (2), Söding (74), Cholodny (15), Dijkman (21), van Overbeek (55)]. In the case of phototropism the process is complicated by a decreased sensitivity of the cells to growth substance in the light (55), and by the fact that the substance is produced in leaves and other green parts in the light [Thimann & Skoog (84)].

The first attempt at chemical study of the substance was that of Nielsen (52), who found that the medium on which *Rhizopus suinus* and other molds had grown contains considerable amounts of the active substance. It is also produced by various bacteria [Boysen-Jensen (10)] and is present in yeasts (53) and higher fungi (54).

Nielsen found the substance to be soluble in ether and to be active in high dilution, 1/60  $\gamma$  of impure syrup causing a definite curvature in *Avena* coleoptiles. Dolk & Thimann (23), also using *Rhizopus* culture medium, found that the substance is extracted by ether only from acid solutions, i.e., the active substance is an acid. By assaying ether extracts made at different pH's, the dissociation constant of the acid was found to be  $1.8 \times 10^{-5}$ , i.e., about equal to that of acetic acid. Its activity is readily destroyed by mild oxidizing agents, such as hydrogen peroxide and the organic peroxides in ether, so that the acid must be an unsaturated one.

Study of the conditions of production of the substance by *Rhizopus* [Bonner (3), Thimann & Dolk (82)] showed the yield to be proportional to the amount of aëration applied, almost no production taking place under anaërobic conditions. The production was also found to be dependent on some specific constituent of the peptone in the medium, and this constituent was not present in all peptones. Similar conditions appear to hold for the production of the hormone from other media and by other molds [Sakamura & Yanagihara (59), Boysen-Jensen (11)]. The substance is both produced and destroyed by *Rhizopus* (82), so that optimal conditions must be selected in order to obtain good yields. The explanation of some of these observations was given later (see below).

At the same time, however, Kögl, Haagen-Smit & Erxleben (36, 37) found the active substance to be present in large quantities in human urine. The bicarbonate-soluble fraction of the ether extract of urine was extracted with petroleum ether, the residue precipitated with lead acetate from weakly alkaline 70 per cent alcohol, precipitated as the calcium salt and finally heated with acid methanol. This, instead of giving an ester, produced what turned out to be a lactone. The product was distilled *in vacuo*, when the bulk of the active substance distilled at 125 to 130°, and yielded crystals of the acid  $C_{18}H_{32}O_6$ , m.p. 196° (named "Auxin A"), or else of the lactone  $C_{18}H_{30}O_4$ , m.p. 173°. Both acid and lactone were active. The activity was such that  $3 \times 10^{-8}$  mg., applied in agar, under the conditions used, gave 10° curvature on the test *Avena* coleoptiles. This was defined as one *Avena-Einheit* (AE). Later determinations (38) showed that there was a fluctuation of several hundred per cent in the curvatures given by a fixed amount of substance. These fluctuations occurred within twenty-four hours and probably had an electrical, or at least a climatic, basis. A mean value of  $2 \times 10^{-8}$  mg. was taken as the weight of one AE.

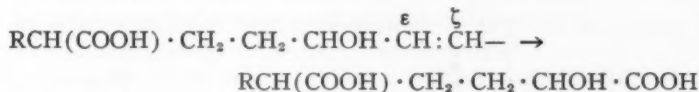
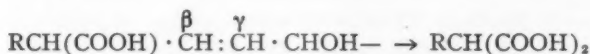
The definition of units, however, calls for a knowledge of the important variables and of the nature of the test. Thus, Went (95) showed that while plants grown in water showed the same sensitivity as those grown in earth, the range of proportional response was wider with the latter. The curvature is not proportional to the total amount of substance applied, as was first thought, but to its concentration. Van der Wey (101) found the curvature to be nearly independent of the size of the agar block. He also made slight alterations in the technic (100). Thimann & Bonner (80) removed the blocks after the test was over and applied them to fresh decapitated coleoptiles. The amount of growth substance which passed into the plant during the test was thus determined, and by using blocks of different sizes it was proved that the curvature is proportional to the concentration of the active substance. The rate at which the substance passes into the plant is proportional to its concentration in the agar block at any moment. By this means the units of the Utrecht and Pasadena laboratories were compared; one *AE* weighs 2.5 times as much as one "plant unit."

The action of the growth hormone is completely nonspecific. It has been shown to control elongation in coleoptiles of various grasses, hypocotyls of lupin, radish, and sunflower (2, 27), flower-stalks of tulip and daisy (74, 87), stem internodes of *Tradescantia* (88) and broad bean (84), and the veins of some leaves; the swelling of the gynostemium of some orchids on pollination is due to the same substance diffusing out of the pollen grain [Laibach (47, 49)].

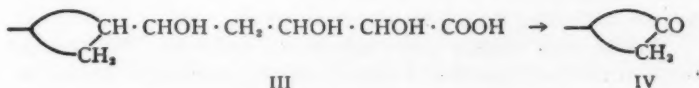
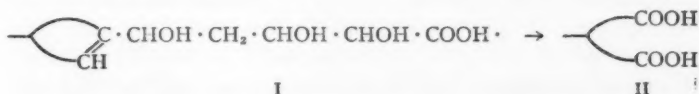
Not only do these growth hormones occur widely in plant tissues, but they are also present in some animal tissues. Maschmann & Laibach (51) and Kögl, Haagen-Smit & Tönnis (41) showed that the amounts in actively growing carcinomas are greater than in other tissues. The latter authors also found bios to be present in larger amounts in cancerous than in normal tissue. Since, however, according to Fischer (quoted in 41), neither auxin A nor B has any growth-promoting effect on tissue cultures, there is no reason to suppose they play any part in the growth of the cancer cells. Kögl, Haagen-Smit & Erxleben (40) found small amounts of auxins in many animal tissues and in blood.

*Chemical nature of the hormones.*—The chemistry of the active substances has been largely elucidated by Kögl and co-workers at Utrecht. The acid and lactone, mentioned above, were shown to have but one double bond, and hence one ring. The rate of mutarotation

of the lactone, compared with Haworth's data, indicated the hydroxyl to be in the  $\delta$ -position. The two remaining oxygen atoms were also shown to be in hydroxyl groups (39). Oxidative degradation gave rise to a  $C_{13}$  dicarboxylic acid which contained no hydroxyl groups and did not lose carbon dioxide on heating. Similar oxidation of the hydrogenated derivative,  $C_{18}H_{34}O_6$ , which is biologically inactive, yielded a neutral  $C_{13}$  ketone (38, 43). The facts are interpreted as follows: the auxentriolic acid ("auxin A") is not enolic in nature, so that the double bond cannot occupy the  $\gamma:\delta$ -position; it cannot occupy the  $\beta:\gamma$ -position since then the  $C_{13}$  acid would be a malonic derivative and would lose carbon dioxide on heating; it cannot occupy the  $\alpha:\beta$ -position since oxidation would then yield a keto-acid; it cannot be beyond the  $\delta$ -carbon atom, since the  $C_{13}$  acid contains no hydroxyl group.



Hence the two carboxyls of the oxidation product must both be formed anew and the original carboxyl must be lost with the five carbon atoms split off. In order for both the new carboxyls to remain in the molecule the double bond from which they arose must be in the ring. The behavior of the hydrogenated derivative, III, confirms this; it gives only the ketone IV on oxidation. Auxin A therefore has the structure shown in I.





ing the active substance from urine, which led to the working up of still larger volumes [Kögl, Haagen-Smit & Erxleben (44)]. Under these conditions it appeared that the bulk of the activity in the eluate could not be purified by the methods previously found satisfactory. The active substance was largely destroyed on attempting to lactonize, and other methods, involving only precipitation and solution, were therefore substituted in the later stages. These led readily to the separation of another active substance, which turned out to be identical with  $\beta$ -indolyl-acetic acid. This substance was isolated from fermentation products by Salkowski (60) and was shown by Salkowski to be also present in urine (61). Its activity per milligram is little less than that of the  $C_{18}$  compounds, and is not due to an impurity, because the synthetic product is fully active. It was also found, and confirmed by Thimann & Koepfli (83), that the corresponding propionic and carboxylic acids are completely inactive, as is also indole itself. Tryptophane, as purchased, usually possesses slight activity, due almost certainly to its content of  $\beta$ -indolyl-acetic acid. The activity of other related compounds is still under investigation, but preliminary results show that a number of related substances possess activity.

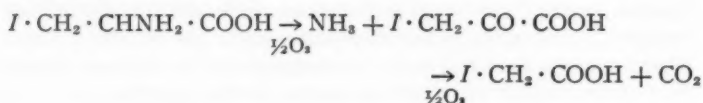
The activity of  $\beta$ -indolyl-acetic acid explains the previously observed fact (40) that urine of a few individuals is of very much higher activity than the normal range. This was shown by isolation from such a urine of  $\beta$ -indolyl-acetic acid in yields as high as 32 mg. from 15 liters (44). The supply of  $C_{18}$  auxins in the urine is derived from the food, principally from fats in which the auxins appear to be present as esters, inactive in growth promotion. Kögl, Haagen-Smit & Erxleben (40) found the auxin content of urine to increase greatly after ingestion of salad oil or butter, but not after ingestion of hydrogenated fats. Correspondingly the action of lipase on arachis oil resulted in the appearance of definite growth-promoting activity in the products.

Since the molecular weight of the growth substance from *Avena coleoptiles* [Went (95); confirmed by Kögl, Haagen-Smit & Erxleben (44)] is around 350, the active substance in these plants is probably auxentriolic acid and not  $\beta$ -indolyl-acetic acid. This was confirmed by the stability of the substance to acid and its destruction by alkali (44); auxin B is destroyed by both treatments while  $\beta$ -indolyl-acetic acid, in common with other indole derivatives, is destroyed only by acids.

However, the active substance produced by *Rhizopus* and *Aspergillus* cultures also shows sensitivity to acid (45). This raised the



possibility that these substances are actually  $\beta$ -indolyl-acetic acid and not the  $C_{18}$  auxins. From yeast plasmolysate small amounts of  $\beta$ -indolyl-acetic acid, representing a large part of the activity originally present, could in fact be isolated by Kögl & Kostermans. That the active substance produced by *Rhizopus* is also, almost certainly,  $\beta$ -indolyl-acetic acid was shown by the writer (79); following purification, the minute amount of substance obtained was identified by the characteristic and very sensitive color reactions, by its distillation temperature *in vacuo*, acid-sensitivity, and many other properties. The substance is probably produced from the tryptophane in the peptone which forms the basis of the culture medium; a peptone from which tryptophane was almost absent gave only very small yields of growth substance (82). This also explains why the yield is proportional to the extent of aëration (82), since oxygen enters into the reaction:



(where  $I$  = indole nucleus). The change is a typical oxidative deamination, and has been recorded for various microorganisms. Those other amino acids which allow significant yields of the active substance [Boysen-Jensen (11)] are in each case those which can most readily be converted to indole acetic acid (79).

*Transport.*—It was mentioned above that in the coleoptile the substance migrates from tip to base (95), and this direction also holds in other plant material (21, 84). Van der Wey (101) has shown that the migration has a strictly morphological polarity; the substance moves from tip to base irrespective of gravity, while in the opposite direction practically no transport occurs. The substance is also transported against its own gradient, being carried from agar blocks of lower to those of higher concentration (102). The amount of substance transported through a coleoptile section in unit time is independent of the length of the section, so that the transport mechanism is not that of diffusion. If the plants are etherized, transport takes place equally in the "normal" and "inverse" directions; after recovery from the narcosis, polarity is again established. All these findings point to the essentially "vital" nature of the transport. In general it resembles the transport of objects along a moving band; the band

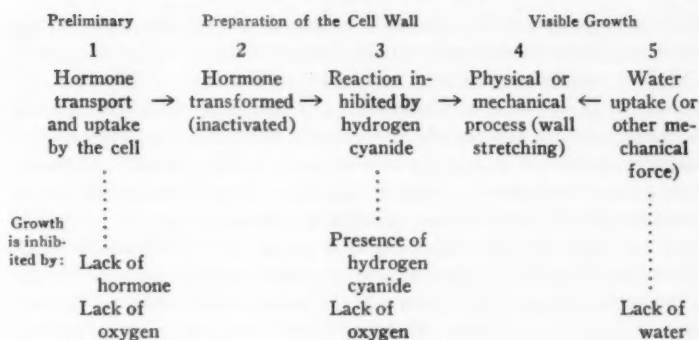
goes at constant speed so that the number of objects arriving at the end per unit time is independent of the length; if not removed from the end the objects continue to pile up (transport against the gradient).

*Mechanism of the action.*—It has been shown by Heyn (32) that the supply of growth substance to coleoptiles results in an increase of the irreversible part of the extensibility; they are more readily curved or stretched by the application of weights, i.e., more plastic. The same thing was shown in another way by Söding (73). The reversible, elastic deformability is little changed by the action of the growth substance, although it apparently also shows a very slight increase. There thus seems little doubt that the increase of plasticity of the wall is the primary factor allowing growth, the expanding force being osmotic. Heyn (33) has also shown that the same phenomenon occurs with lupin hypocotyls and the flower stalks of tulips, and that the plasticity change may occur even if subsequent growth is prevented by not supplying water, i.e., it precedes, and does not result from, the actual growth. Further, after geotropic stimulation, Dijkman (21) found that the concentration of growth hormone at the lower side, discovered by Dolk (22), is established in half an hour, while Heyn finds the increase in plasticity of the lower side to appear at about the same time.

The extension of coleoptiles is independent of the laying down of wall tissue, since the latter process, which leads to thickening, practically ceases at 0°, while extension continues [Heyn (32), Heyn & van Overbeek (34)]. Similarly, in water containing small amounts of growth substance, sections of coleoptiles may continue to extend at 0° without increase in weight, while if fructose is added the increase in weight may either balance the extension, weight per unit length remaining constant, or may exceed it, weight per unit length being increased [Bonner (5)]. The differences are controlled by temperature. Growth cannot, therefore, occur by a primary process of apposition or intussusception, as was supposed by the older workers; the primary process must be some kind of release of the forces binding together the components of the cell wall. An examination was therefore made of the stoichiometrical relations between the growth hormone applied and the constituents of the cell wall [Thimann & Bonner (81)]. Since cellulose, which is of a crystalline, and therefore more or less rigid, nature, comprises about half of the cell wall material, particular attention was paid to this constituent. Determinations,

by the biological assay, of the actual amounts of growth hormone entering the coleoptile were compared with measurements of the total increase in length, and with chemical analysis of the cell-wall material. The results show that for every molecule of growth hormone entering the growing coleoptile, about  $3 \times 10^6$  hexose residues are laid down in the form of new cellulose. Even if the cellulose exists as micelles there would be 150 new micelles laid down per molecule of growth hormone entering. Correspondingly high figures for other constituents of the wall, such as pectin, hemicellulose, and protein make it impossible that the growth substance can combine chemically with any of the constituents which determine the structural rigidity of the material. The increased plasticity cannot therefore be ascribed to chemical combination with the wall. Further calculations (81) show that there is insufficient growth hormone to form even a monomolecular layer on the new walls, so that any kind of action through increasing the permeability is also highly improbable. Hence the action probably involves some indirect or catalytic type of process. This is supported by Bonner's finding (4) that elongation only occurs in oxygen and is suppressed by hydrogen cyanide in those concentrations which inhibit the respiration of the tissue. The oxygen requirement explains the older observation [van Amejden (1)] that oxygen is necessary for the response to light and gravity.

When plant tissues are crushed the growth hormone is inactivated, presumably by the oxidase system. Stark (75), for instance, could obtain no growth-promoting action from crushed coleoptile tips, etc. However, by crushing and extracting together in chloroform the substance may be recovered, and this allows direct determination of its concentration in the tissue [Thimann (78)]. Its concentration decreases from the tip towards the base of the coleoptile, as would be expected. By similarly extracting coleoptiles to which various concentrations of growth hormone had been applied after decapitation, Bonner & Thimann (7) showed that the growth hormone disappears continuously during growth, the growth produced being proportional to the amount of hormone which has disappeared. Since the disappearance is not prevented by hydrogen cyanide it must involve a process other than that which is inhibited by hydrogen cyanide. Growth substance which has been applied in excess and has then disappeared is not available for subsequent growth. The growth process can therefore be presented, schematically, as follows, all the preliminary reactions being required to occur together:



It is of interest that apparently the free acid, and not the salt, must be present in order for growth promotion to result. When it is applied in agar, buffered at pH 7, no curvatures are produced by active solutions (23). Subsequently, Strugger (77) found that when plant tissues are exposed to acid on one side they give rise to very large curvatures. These curvatures are due to an actual acceleration of growth. Correspondingly, Bonner (6) found coleoptile sections to grow much faster than normal when infiltrated with dilute acid. The growth acceleration is accompanied by an increase of plasticity, is inhibited by hydrogen cyanide in those concentrations which inhibit growth acceleration, and is about proportional to the concentration of free acid which would be produced from the growth hormone already present in salt form. On these grounds it is practically certain that the acid growth, and correspondingly also the acid curvatures, are due to the setting free of the auxin acid from its salt form. The importance of the acid nature of the auxins for their polar transport, particularly if electrolytic processes are involved, has been emphasized by Went (97).

*Growth of roots.*—While the three growth substances discussed above promote the elongation of coleoptiles, stalks, and other above-ground parts of plants, they appear to inhibit the growth of roots. Thus Cholodny (14) showed that coleoptile tips or even root tips themselves [(18), Keeble, Nelson & Snow (35)] inhibit the growth of decapitated roots to which they have been applied; the secretion of root tips into gelatin or agar promotes the growth of coleoptiles (12) and inhibits that of roots [Hawker (31)]. Inhibition of growth of roots on immersion in growth-substance solution was shown by Nielsen (52) with impure, and by Kögl, Haagen-Smit & Erxleben (44)

with pure preparations. Growth substance is present in root tips, however, since it diffuses out into dextrose agar [Boysen-Jensen (12)] or can be extracted by chloroform [Thimann (78)]; this explains the observation of Cholodny (15) that roots grow faster after decapitation. In geotropism the growth hormone becomes concentrated on the lower side of the root where it inhibits growth and therefore causes downward curvature (12, 31). Decapitated roots do not react to gravity, but recover sensitivity when the tips, or coleoptile tips, are replaced [Keeble, Nelson & Snow (35); Cholodny (14)]. Similarly, decapitated coleoptiles recover their sensitivity after the application of root tips [Cholodny (17)]. In each case it follows that the root tip produces growth substance which controls the tropistic response of the stump. Isolated and in culture, root tips can grow indefinitely [White (103)], so that if they need hormones for their growth these must be in the media used. The root tip does not appear to synthesize growth hormone when isolated, unless it is supplied with nutrient [Cholodny (19)]; otherwise it does not set free into agar more than can be extracted from it in the first place [Thimann (78)]. Both the mechanism of the growth of roots and its hormonal control are far from understood.

#### ROOT FORMATION

Although it was postulated by Sachs in 1882 (58) that special substances other than nutritive materials are concerned in root formation, knowledge of the hormonal control of the process has been limited to the last few years, it having been for a long time thought that nutritive factors alone were responsible. The experiments of Vöchting (91) showed that the neoformation of roots is of a polar nature, being restricted to the morphological bases of cuttings, independent of gravity. However, some roots being derived from pre-existing root germs are not subject to the same rule. The work of van der Lek (50) showed that the presence of buds or leaves on a cutting promotes the formation of roots at the base, the roots appearing on the same side of the stem as the buds. He supposed hormones to be responsible but made no specific deductions as to their nature or method of action. Went (96) and Bouillenne & Went (8), whose publication includes a review of the earlier work, established that a definite root-forming substance is responsible: first, the grafting of leaves on to woody cuttings led to the formation of roots at the base of the cutting [somewhat similar experiments were made with *Bryophyllum* by Went

(94)]; second, water in which cut leaves had been placed had the same effect; third, boiled malt diastase and an extract of rice polishings were also found to be active. The stimulative substance is therefore thermostable. The action of diastase and rice polishings shows it to be present in seed, and the above evidence also indicates it to be produced in leaves. Since leaves of one species act on cuttings of another, the substance is non-specific. Other experiments show that it is stored in buds and to some extent in woody stems themselves, and that it is formed in the light. The polarity of root formation mentioned above is therefore due to the polar transport of the root-forming substance, "Rhizocaline," which in this respect is similar to that of the auxins.

The activity of the substance having been established, a suitable method for its assay was worked out by Went (98) using pea cuttings (whose cotyledons are below ground) grown in the dark and deprived of buds, i.e., as free as possible from the active substance. Using this method, a chemical investigation was undertaken by Thimann & Went (85), who found that while the active substance was present in numerous extracts, the crude auxin preparations from *Rhizopus* and from urine were by far the best sources. The activity was seen to run parallel with that for cell elongation through the various stages of purification, so that the substance must be similar to auxin. Both are unsaturated organic acids, of similar dissociation constant and solubility, and similar sensitivity to mild oxidizing agents. It was then found that the crystalline auxins prepared by Kögl and co-workers were also active in root formation, and that they lose their activity on oxidation, parallel to the loss of growth-promoting activity. Finally, it was found [Thimann & Koepfli (83)] that synthetically prepared  $\beta$ -indolyl-acetic acid has the same root-forming activity (of the order of  $10^5$  root units per mg.), the activity varying somewhat from test to test. Hence the root-forming hormone is identical with the cell-elongating hormone (auxins), all three auxins being also active in root formation. While this would seem to show that the fundamental process catalysed must be the same in cell elongation as in root formation, it must be remembered that the latter is of course a composite, probably involving several complete processes. Oxygen is necessary for the neoformation of roots [Zimmermann (104)]; this may be compared with its rôle in cell elongation. The formation of roots probably involves substances in addition to the hormone above discussed, at least in certain cases. In this connection it is of interest that

ethylene, acetylene, carbon monoxide, and other gases strongly promote the formation of roots on the intact plant [Zimmermann, Crocker & Hitchcock (105)]. The nature of the action of these gases is unknown; van der Laan (46) showed that, in *Avena* and *Vicia*, ethylene reduces production of the growth hormone, but this can scarcely cause root formation. In conclusion it may be repeated that root formation has apparently nothing to do with the elongation of roots; indeed, the substance that stimulates the former inhibits the latter.

#### BUD INHIBITION

It has been known for a long time that the terminal bud of a young plant inhibits the development of lateral buds lower down on the stem; when the terminal bud is removed the lateral buds, which normally would have remained dormant, begin to develop. Not only the terminal bud, but also to a lesser extent the leaves [Dostál (24), Snow (69)] exert an inhibiting effect. An indication that the inhibition is due to a substance transported to the lateral bud through the stem was given by Snow, who showed that inhibition could pass across a discontinuity (67) or across a dead stretch of stem (68). It has now been shown not only that this is the case, but further that the inhibitor is identical with the growth-promoting hormone described above [Thimann & Skoog (84)]. It was first shown that in *Vicia faba* the rate of production of growth hormone is very high in the terminal bud and smaller in the leaves. It is not produced in appreciable amounts in dormant lateral buds, but when these buds begin to develop production of growth hormone takes place in them also. Thus, those organs which inhibit bud development also produce growth hormone, the production being about proportional to their inhibiting activity. Finally, the terminal bud was removed and growth hormone applied at regular intervals in its place; this led to complete inhibition of the buds. When application of the auxin was stopped the buds at once began to develop. In later experiments, using peas (66), it was shown that the crystalline auxins of Kögl, Haagen-Smit & Erxleben inhibit bud development as completely as do the impure *Rhizopus*-auxin preparations first used, so that there is no question of a second, special, inhibiting factor being involved. Recently, Uhrová (86) was able to inhibit bud development in *Bryophyllum* by the application of agar on which leaves of the same plant had stood for some time, while Laibach (48) showed that the application to decapitated *Vicia* of orchid-pollen grains will also inhibit bud development. Although in neither of these cases was the



active substance identified there is no reasonable doubt that here, too, it is the growth hormone.

In inhibition, as in root formation, it has thus been proven that the pure auxins, or growth hormones, are the active substances. Thus these substances are involved in at least three processes, cell elongation, root formation, and bud inhibition. It is remarkable, furthermore, that both the  $C_{18}$  substances and the indole derivative are active in all three functions. It remains to be seen whether other active substances share all three activities. In any case the findings suggest that the mechanism involved must be extremely fundamental in nature.

#### MISCELLANEOUS

*Cell division.*—The first plant hormone to be demonstrated was the substance, produced in wounded tissue, which stimulates cell division [Haberlandt (28)]. Although of considerable interest, the matter has not received chemical attention since the work of Wehnelt (92) who found the active substance in wound tissue to be extractable with alcohol, and found at the same time that many products, even including agar, had definite activity. It has now been made highly probable, if not proven, by Snow (71) that cell division in the cambium is also stimulated by a substance which passes from tip to base in the intact plant.

The effect of sulphydryl substances in promoting cell division in the onion root, as well as in other material, may also be mentioned [Hammett (29)]. The effects are reported to be small and confirmations have not yet appeared; for animal material there have been definite contradictions.

*Do sex hormones act on plants?*—It was claimed by Schoeller & Goebel in 1931 (62) that technical progynon, and, in later experiments, the pure substance (63), hastens the flowering of hyacinths and of *Calla*; the preparations were added to the water in which the bulbs were placed. Harder & Störmer (30) now find that application of pure progynon to *Narcissus* for seventy-four days causes no difference whatever in the time or quality of flowering. Virtanen, Hausen & Saastamoinen (89), using the same methods, temperature, etc., as Schoeller & Goebel and even using Schoeller's progynon preparations, also obtained negative results. Furthermore, progynon was without effect on other plants. Euler & Burström (26) also report negative experiments, so that the original positive results must be ascribed either to individual differences and insufficient controls, or else, pos-

sibly, to unknown experimental factors. It will be remembered that Butenandt & Jacobi (13) and Skarzynski (65) were able definitely to isolate female sex hormone from plant sources.

*Other effects.*—As opposed to the negative results with progynon, Virtanen & Hausen (90) report that the injection of yeast extract causes a real stimulation of flowering in peas and beans; details are not yet to hand. Laibach (48) finds that the application of orchid-pollen grains to *Coleus* petioles from which the leaf has been cut off prevents the falling off of the petiole; the effect is ascribed to the growth-hormone (auxin) content of the pollen. Application of pollen extract or urine, in lanolin paste, causes leaves to roll up, due to one-sided growth (48). Doubtless other phenomena caused by growth hormone will come to light in the near future.

#### GROWTH SUBSTANCES FOR FUNGI

Our knowledge of the requirements of special growth-promoting substances by fungi is still slight. Since a hormone is understood to be a substance which is produced within the organism on which it acts (cf. Bayliss & Starling's original conception of a chemical messenger), the growth-promoting substances of the lower plants, which have to be supplied from without, must be regarded rather as vitamins.

It was found by Nielsen that the culture medium on which *Rhizopus suinus* had grown contained, besides auxin, substances promoting the growth of yeast (110) and of *Aspergillus niger* (111). Addition of 1 per cent of the sterilized medium to the culture of *Aspergillus* on synthetic medium increased the weight of mycelium about 50 per cent. By increasing the additions, a yield of mycelium up to nine times the control was obtained, while the formation of conidia was also promoted. The optimum pH for *Aspergillus* depends on whether the growth-promoting factor is present; in its presence the optimum initial pH is 6 to 7, in its absence about 2.5 (112); the normal increase in acidity of the medium is largely prevented, due, according to Bünning (106), to stimulation of the uptake of nitrate ions from the medium. The active substance is insoluble in ether, being thus separated from the auxins; it is slightly soluble in 90 per cent alcohol, and is stable to hydrogen peroxide and to heat [Nielsen & Hartelius (113)]. Thus it is entirely different from the growth substance of higher plants, and is termed growth-substance B (not to be confused with auxin B).

Further study showed that the autoclaving of the medium alone produces a marked growth-promoting effect, the substance being ap-

parently formed from glucose, tartaric acid, and some constituent of the filter paper (114). Fructose or arabinose, but not inositol, mannitol, or glycerol, could replace glucose, while various organic acids could replace tartaric acid. The active constituent of the filter paper resembles, but is not entirely replaceable by, zinc, and it appears to function as a co-growth substance, increasing the effectiveness of the added growth-substance B. Growth-substance B also occurs in urine, and here also its effectiveness is promoted by addition of zinc or filter ash [Hartelius (108)]. The amount present varies with the total solids of the urine, and is unaffected by pregnancy; it is, however, dependent on the food intake, the concentration in the urine being greatest after meals, just as was found by Kögl and co-workers for auxins. The nature of the active substance (or substances) remains unknown.

In another case, however, the growth-promoting substances have been identified. It was shown by Schopfer (116) that *Phycomyces blakesleeanus*, which will not grow at all on a synthetic medium made up with glucose, grew if maltose were used instead. The effect was not due to the sugar itself, but to an alcohol-soluble impurity, present only in Kahlbaum maltose, and apparently of nitrogenous nature. Substances of similar activity are present in wheat germ and in yeast (117), and their action appears to allow both growth and zygote formation. Wassink (119) also found that a thermostable vitamin-like substance, present in yeast, was necessary for growth of *Phycomyces* to occur on synthetic media. As a result of partial purification he concluded that the active substance was of the bios type.

However, it has now been shown by Schopfer (118) and Burgeff (107) that the active substance is vitamin B<sub>1</sub>; as little as 0.2γ per cc. of the pure substance of Windaus allowed marked growth with much zygospore formation. According to Schopfer, vitamin B<sub>2</sub> (Kuhn) is also active, its activity being only 20 per cent of that of B<sub>1</sub>. Burgeff (107) also found that two other molds, *Chaetocladium macrosporum* and *Parasitella simplex*—both normally parasitic on *Mucor* mycelium—respond strongly to additions of vitamin B<sub>1</sub>. Bünning (106) found vitamin B<sub>2</sub> to have a small growth-promoting effect on *Aspergillus niger*, while B<sub>1</sub> was practically without any; on the other hand, both stimulated the uptake of nitrate ions from the medium.

Other miscellaneous effects have been recorded. Okunuki (115) found red torulae to produce both growth-inhibiting and growth-promoting substances for molds; the growth-inhibiting substances

are soluble in ether and acetone, and the growth-promoting, insoluble. Williams & Honn (120) found yeast extract to promote the growth of various molds on different synthetic media. Lepeschkin (109) showed that vitamin-B concentrates promote the growth of *Penicillium glaucum*. In general, it appears that the growth-promoting substances for molds all belong to the vitamin-B group.

### Bios

Since the substances promoting the growth of yeast have been reviewed in 1925 by Tanner (138), and in 1930 by Miller (131), only recent developments need to be added.

As to methods, Lacroix (130) and Almoslechner (121) have introduced the determination of the generation time, by counting cells in drop cultures of synthetic medium, with and without addition of active extract.

Bios is frequently present in cane sugar, and can be extracted therefrom with alcohol, an indication that great care must be exercised in making up control solutions [Hall & James (128)]. Bios is produced by the *Avena*-coleoptile tip along with the plant-growth hormone [Euler & Philipson (127)]. It is present in large amounts in the ether-insoluble residue of *Ranunculus* leaves [Boas (122)], and is also formed by various molds [(127), Nielsen (133)].

It was found by Schwartz & Kautzmann (136) that wort, after treatment with yeast, has a decreased bios activity. Nielsen (134) has shown that this is due to absorption of the bios by yeast, without any growth being involved. Most of the absorption takes place in the first four hours; the amount of bios absorbed varies with the time of contact and the amount of yeast used. This is reminiscent of Eastcott's work on the absorption of inositol, which could be largely recovered from the yeast afterward (125).

It will be remembered that Eastcott found one constituent of bios to be *i*-inositol, but subsequently neither Narayanan (135), Williams and co-workers (140), nor Edwards (126), could find any appreciable effect of inositol, either with or without other bios preparations. Considerable interest, therefore, attaches to the findings of Buston & Pramanik (124) on the growth-promoting factors for the yeast-like organism, *Nematospora*. In this work the active material from an extract of lentils could be separated into two fractions, neither of which had much activity alone. One of these fractions was *i*-inositol; pure *i*-inositol from other sources was equally active as a supplement

to fraction II. Buston & Kasinathan (123) further showed that *d*- and *l*-inositol, quercitol, and quebrachitol were all inactive. Fraction II appears to be a nitrogenous organic acid, and is difficult to characterize; it was not obtained pure.

Part of the difficulty about inositol appears to rest on differences between yeasts, since Stantial (137) has again found, as has repeatedly been reported in the past, that some yeasts do not respond to inositol, with or without other bios fractions, while others respond as did those of Lucas and of Eastcott. However, Williams & Saunders (142) have compared their yeasts with those of Narayanan and of the Toronto workers, and find that in every case, but most clearly when the growth is large, inositol definitely increases the effect of other bios preparations. Their previous failure, and the failure of other workers, to confirm the effect of inositol is ascribed to the fact that larger amounts are needed of inositol than of the other fractions, and that the effect is only well-marked with large growths and with the right supplements. The importance of *i*-inositol is thus apparently re-established. In regard to the failures to detect its action, Miller, Eastcott & Maconachie (132) further point out that some of the purification processes used may not have separated bios II from the last traces of inositol, so that sufficient inositol remained to give the necessary supplement. These authors have been able to separate bios II by adsorption on charcoal into two further fractions, neither of which is very active alone, but which when combined, and inositol added, show very high activity.

As to the chemical nature of bios, the advance has been slow compared with the advances in our knowledge of the higher plant hormones and the vitamins. The bios of Nielsen (134) is, like growth-substance B of fungi, thermostable, ether-insoluble, and stable to hydrogen peroxide. Unlike growth-substance B, however, it cannot be produced by heating together sugars, organic acids, and filter-paper. Partial purifications of bios have been worked out by Narayanan (135) (including a complete separation from vitamin B<sub>2</sub>), and by Edwards (126), including a separation from the fermentation-promoting factor Z. Narayanan's product is stable to acids and bases, including nitrous acid, but is inactivated by hydrogen peroxide. It is apparently a simple nitrogen compound, but was not obtained pure. Williams & Truesdail (139) found that rice-bran extracts, which are rich in bios, can be separated from accompanying toxic compounds by fractional electrodialysis in a 4- or 6-compartment cell. In this way

the bios was separated into two components, neither of which was active alone. One was an acid, migrating always to the cell whose final pH was from 3 to 5. Edwards (126) obtained similar results. Further study of this method, using a variety of biological extracts, resulted in every case in the concentration of the activity on the acid side at a final pH of about 3.6 [Williams *et al.* (141)]. The yeast now used responded to this fraction alone.

The active substance is an ether-insoluble organic acid of molecular weight about 150. The activity is destroyed by nitrous acid or by alkali, but not by hydrogenation. Since the active substance in the various extracts appears to be the same, and is widely distributed in various natural orders from higher animals and plants down to slime molds and bacteria, it was termed "pantothenic acid" (Greek, "from everywhere"). Further, not only the Gebrüde-Mayer yeast which was used, but five other strains all showed a large response to the addition of concentrates of the substance [Williams & Saunders (142)]. One of these yeasts was stimulated by pure vitamin B<sub>1</sub>, particularly when supplemented by pantothenic acid. The acid has not been isolated in the pure state. Isolation of a crystalline bios from egg yolk has been briefly reported by Kögl (129). It contains basic nitrogen and probably a carboxyl group, i.e., it is amphoteric; it is stable to hydrogenation and may be identical with pantothenic acid. Its activity is very high, one part in  $4 \times 10^{11}$  parts of solution having a distinct effect. In the presence of inositol, which alone has no effect on the yeast used, the activity is increased about 50 per cent. The substance is thus a bios II, and further knowledge of its chemical nature will be awaited with interest.

It will be apparent from the last two sections of this review that the field of growth substances for microorganisms is as yet scarcely touched. A considerable amount of work has been done on growth-promoting substances for bacteria and even for protozoa; space forbids its inclusion here. Only as the exact chemical requirements for growth of the different groups of organisms become known will it be possible to understand the mechanism of the growth process itself. It appears that a beginning in this direction is being made in some of the processes of the higher plants, and corresponding developments with the lower plants are to be expected in the near future.

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## IMMUNOCHEMISTRY\*

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The division of the subject is as in Volume II. Space requirements have again made it necessary to eliminate mention of many papers. The period covered is essentially that from December, 1932, to November, 1934.

During this time the literature of immunochemistry has been enriched by three important books. The first, by Landsteiner (1), which is to appear in English,<sup>1</sup> gives impartially and fairly all possible interpretations of the phenomena of specificity with amazingly complete references to the original literature. The second, by Topley (2), provides a stimulating discussion of modern work and theories, emphasizes the importance of the bacterial surface in reactions with antibodies, and the necessity of the evaluation of the probable error before a conclusion is drawn, but abandons the last principle in accepting the "optimal proportions" method as a standard for precipitins and agglutinins. The third, a monograph by Marrack (3), gives clearly and with graphs, which in some cases improve on the original papers, a critical survey of the physics and chemistry of antigens, antibodies, and their interaction. To the benefit of his reader, Marrack does not hesitate to speculate, and is thereby enabled to provide a most plausible and suggestive picture of specific combination, precipitation, and agglutination, with which, however, the reviewer is not wholly in accord. In a monograph by Abramson (4) there is also a chapter on "bacteria, antibodies, viruses, and related systems." For a review on antigens and a lecture on immunochemistry see 4a and 4b respectively.

### THE CHEMISTRY OF IMMUNE SUBSTANCES

#### NATURALLY OCCURRING ANTIGENS

From precipitin tests with native serum albumin (A), heat-denatured or acetone-denatured serum albumin (B), and B in which the denaturation had been reversed (C), and the corresponding antisera,

\* Received January 16, 1935.

<sup>1</sup> Private communication from the author.

Miller (5) concludes that B is markedly changed in its antigenic properties, although anti-C serum reacted almost equally well with A, B, and C. A and C were indistinguishable immunologically. The differences in the case of B were accentuated by a long-continued denaturation process. Hewitt (6), however, considers evidence lacking that the "reversed" albumin had ever been denatured. It is to be hoped that Anson and Mirsky, who have worked so much with the reversal of denaturation, will clear up these difficulties. Hektoen & Welker (7) have shown that plasma, adsorbed on  $Al(OH)_3$ , stimulates a lasting production of antibodies differing by the presence of anti-fibrinogen from those produced by serum. These workers (8) have also studied the antigenic properties of "Arndt" and Bence-Jones urinary protein fractions. Ronse (9) has shown that the antibody to human casein does not precipitate the casein of cow's milk, while Lewis (9a) found goat casein to be isoantigenic by the complement-fixation reaction. Precipitin formation was not mentioned. Ratner & Gruehl (10) have given evidence that intact protein could pass the intestinal walls of some of the guinea pigs tested. Block & Brand (11) have shown that 90 per cent of brain protein has the properties of nucleoprotein, and that these fractions of beef and pig-brain proteins are antigenically distinct. Schwentker & Rivers (12) have found that fresh emulsions of homologous brain are scarcely antigenic, but autolyzed emulsions and those infected with vaccine virus produce complement-fixing antibodies which are partly organ specific. The specific antigen is more abundant in the white matter and parallels the myelin content. Jorpes (13) has studied the fibrin-like "stromatin" in horse and sheep red cell stroma.

Enzymes have again been shown to be antigenic by Ten Broeck (14), who found that trypsin, chymotrypsin, and chymotrypsinogen actively sensitize guinea pigs with some degree of specificity. The last two do not readily form precipitins in rabbits. Walton & Segura (15), however, found no evidence of antipain formation in dogs, the enzyme precipitating both normal and "immune" dog serum.

As for the vegetable proteins, Ishigami (16) has found rice and soy bean glutelins to be antigenic, but the partial hydrolysis products of the latter are immunologically inactive. Additional evidence has been presented that the allergens of ragweed pollen are proteins (17).

The bacterial antigens have received much attention. In the *Salmonella* group White (18) has continued his antigenic analysis, finding that in *B. proteus* X 19 the "O" or heat-stable somatic complex

contains an alkali-labile factor responsible for the "O" agglutination in homologous antiserum and an alkali-stable factor<sup>2</sup> responsible for the cross reaction with typhus serum. From members of the group, in addition to the "Q" protein, White has removed with a higher acid-alcohol concentration a "T" protein flocking at pH 7.2, common to the group, and probably not on the bacillary surface. Antibodies to this protein agglutinate "R" and "q" strains but not alcohol-washed "S" or young living cultures. The special serological properties of the q form depend on carbohydrate haptens (20). By extraction of *B. Aertrycke* with 0.25 N trichloroacetic acid in the cold, Boivin and collaborators (21) have isolated an antigenic complex which is split by boiling acetic acid into a non-antigenic specific polysaccharide and a phosphatide, the latter yielding a crystalline fatty acid<sup>3</sup> on further degradation. Other organisms gave analogous products. Similar results with *B. Aertrycke* have been reported by Raistrick & Topley (22). Grasset (23) has detoxified extracts of the typhoid group of organisms with formalin and reported success in the immunization of humans.

Day has continued his study of pneumococcus antigens (24), finding a type-specific antigen which may be extracted with dilute hydrochloric acid, but which is dissociated by alkali or by pneumococcus autolytic enzymes to a species-specific antigen and is then destroyed. An acid-resistant, alkali-labile antigen, yielding protective antisera, was also extracted from very virulent hemolytic streptococci (25). Boor & Miller (26) have studied gonococcus and meningococcus "nucleoproteins" [cf. also Rake & Scherp (27)], finding them almost as toxic as the organisms themselves and failing to obtain evidence of separate endotoxins. Antisera to the proteins contained antibodies reacting with the specific polysaccharides, but since the antigens were not filtered to remove bacterial fragments their presence was not excluded. Similar observations have, however, frequently been made in the reviewer's laboratory in the case of filtered streptococcus and tubercle-bacillus proteins.<sup>4</sup>

Strains of the *Brucella* group studied by Huston, Huddleson & Hershey (28) contained a small amount of a water-soluble "albumin"

<sup>2</sup> Probably a specific polysaccharide according to Castaneda (19).

<sup>3</sup> Unpublished experiments by the reviewer and Menzel have shown that magnesium palmitate is split from the high-rotating polysaccharide of tubercle bacilli by treatment with alkali.

<sup>4</sup> Unpublished experiments.

which disappeared rapidly if enzyme action was not avoided, and 4 to 7 per cent of a water-soluble nucleo-protein which split off nucleic acid when treated with alkali.

Tomcsik & Szongott (29) have given more study to the type-specific "protein" isolated from the capsule of the anthrax bacillus, which, however, fails to give most of the protein reactions. Its chemical behavior appears to the reviewer to be remarkably like that of the specific polysaccharide of Type I pneumococcus.

Analyses of cholera-vibrio proteins are reported by Linton and co-workers (30). Krueger (31) has given a method of disintegrating bacteria by extraction with buffer in a mechanical grinder. This was adopted by Huston *et al.* (28), using ice to minimize enzyme action and water instead of buffer.

Proteins of a human strain of tubercle bacillus have been fractionated by Heidelberger & Menzel (32) by the method originally used for *Streptococcus*. Fractions of differing optical rotation and phosphorus content were obtained, and of these the neutral extractable protein was antigenically distinct from the portion extracted by strongly alkaline media. All fractions showed a strong tuberculin activity, differing in this respect from the relatively inactive "albumin" and "globulin" isolated from the bacillus by Gough (33) without precautions to avoid enzyme action. Pedersen-Bjergaard (34) has found that tubercle-bacillus phosphatides prepared according to Anderson stimulate the production of complement-fixing antibodies. Since most of the nitrogen in the phosphatides could be liberated as ammonia it was considered that the antigenic properties could not be due to protein. Gough (35) has purified the skin-active material in tubercle-bacillus filtrates by precipitating benzoic acid in the liquid, drying the precipitate, and removing the benzoic acid with acetone. No analyses are given. Spiegel-Adolf & Seibert (36) found that nucleic acid could be removed from purified tuberculin by ammonium sulfate fractionation without impairing its activity. Seibert & Munday (37) found no marked differences in the composition of ammonium sulfate-purified tuberculins from human, bovine, avian, or timothy strains. The presence of polysaccharide influenced the nitrogen partition. Cystine nitrogen was lowest in the human tuberculin. Schaefer & Sandor (38) found that the proteins in tubercle-bacillus culture fluid gave rise to complement-fixing antibodies distinct from those formed in response to the bacillary lipoids. Boquet & Sandor (39) found a specificity difference in the proteins of unheated tubercle-bacillus cul-



tures and those of heated tuberculin. Kallós & Hoffmann (40) report that the activity of a polypeptide, " $\beta$ -tuberculin," isolated by ultrafiltration from bouillon cultures or the blood, urine or skin of tuberculous patients is proportional to the tryptophane content.

Pyl (41) has pointed out physicochemical analogies between the virus of foot and mouth disease and proteins and enzymes. Galloway (42) has shown that the fixed virus of rabies is still antigenic after inactivation by the combined action of dyes and light.

Much work has been done with bacteriophage. Schlesinger (43) has effected a far-reaching purification of an anti-*B. coli* phage, getting twenty to fifty times the usual concentration on a synthetic medium. By ultrafiltration, removal of the phage from the membrane, and fractional centrifugation at high speed, the phage was obtained free from agglutinin as a sulfur-like solution with properties much like those of nucleoprotein. Fifteen mg. failed to show respiratory or fermentative activity. The particles were usually agglutinated by antiserum, but could be neutralized without agglutination. Andrewes & Elford (44) found that the presence of phage-antiphage complex failed to retard neutralization of fresh phage by antiphage, which was taken as evidence against a mass-law explanation of the reaction. There was also no Danysz effect. It was considered that different phage particles varied in their resistance to antibody, since a given amount of serum neutralized 95 per cent of the phage in four hours regardless of the phage dilution. In confirmation of Asheshov & Sertic's observation, the inhibition of phage-antiphage interaction (45) by bacterial extracts has been traced by Burnet & Gough (46) and Levine & Frisch (47) to a specific polysaccharide which Burnet & Gough have found to be alkali-labile with respect to its phage-inhibiting power but not as to its antiserum-precipitating property. Burnet (48) has also studied the chemical inactivation of phage, as have Wells & Sherwood (48a); and Krueger and co-workers (49) have shown that the inactivation by mercuric or cyanide ions may be completely reversed by suitable removal of the inactivating ion, so that the inactivation appears to be like that of an enzyme. Meyer & Taslakowa (50) conclude, however, from the antigenic invariance of phage on substrates of different antigenicity that assimilative activity and formation of new substance are shown.

Bacterial hemolysins have also been studied extensively. In the action of staphylolysin on the red cells of different animals Forssman (51) reported that it behaved much as an enzyme, and could some-

times be found in undiminished amount when lysis was complete. The great difference from lysis by antibody, or immune hemolysin, is stressed. Birch-Hirschfeld (52) has shown that hemolysin and protease run parallel in extracts of *Staphylococci* grown on cellophane agar, but may be separated. Schwachman, Hellerman & Cohen (53) have found that *Pneumococcus* hemolysin behaves like sulfhydryl compounds, being reversibly inactivated by cuprous oxide and organic mercury compounds capable of forming stable mercaptides. Its oxidation-reduction behavior is much like that of urease and papain.

Again there has been much work on the neutralization of toxins by various substances, and many methods, mainly modifications of older methods, have been used for the purification of toxins, but since these have not yet led to the actual isolation and study of a chemically well-defined, pure toxin, they cannot be taken up in a brief review. Only Krestownikowa & Rjachina (54), who described purified erysipelas and meningococcus toxins, have reported for comparison parallel experiments on the broth used. Wheeler (54a) did not find any correlation between protein synthesized and toxicity in diphtheria cultures on synthetic media.

Schmidt (55) has made a detailed study of the mechanism of diphtheria-anatoxin formation by formaldehyde, finding that it differs from "toxoid" in its non-reversibility, stability, and lower amino nitrogen content. Contrary to Burney, it could be prepared from purified toxin with very low concentrations of formaldehyde. Hooker & Follensby (56) have given evidence that scarlatinal toxin may contain at least two separate toxins characterized by different chemical and physical behavior.

#### CHEMICALLY ALTERED PROTEINS AS ANTIGENS

Wormall's views, as against those of Bruynoghe and Adant, on the serological relationships of bromo and iodo proteins, have been confirmed by Finkelstein (57). Hopkins & Wormall (58) have made a study of phenylureido- and *p*-bromophenylureido proteins formed by the action of the aryl isocyanates at pH's not exceeding 9.5. The precipitin and inhibition tests support the view that the free amino groups in intact protein (also the reactive groups with isocyanates) are the  $\epsilon$ -amino groups of lysine. The specificity changes were much like those produced by the introduction of aryl azo groups into proteins. The ureido gelatin derivatives were not antigenic but precipitated antisera to the ureido horse-serum globulins and ureido caseino-

gens. With the ureido amino acids complete inhibition of specific precipitation was obtained only with those from lysine and  $\epsilon$ -amino-*n*-hexoic acid. Somewhat at variance with the above is Lewis' finding (58a) that deaminized casein seemed immunologically identical with casein. Doerr & Girard (59) have found that atoxylazo-racemized egg albumin is not antigenic, but is precipitated by antisera to atoxylazo egg albumin. Reiner (59a) has reported greatly impaired immunizing power for toxin or toxoid put through the coupling process with diazotized atoxyl.

#### HAPTENS

*General.*—As will be seen below, the distinction between haptens and antigens is no longer absolute, but "hapten" is so convenient a term for the portion of an antigen determining a particular specificity that it will undoubtedly continue to be used. Medveczky (60) has proposed a logical but unnecessary and somewhat cumbersome system of nomenclature for the haptens.

Erlenmeyer, Berger & Leo (61) have continued their work on the immunological relationships of substances with equal force fields, improving their technique to meet objections previously raised. They found crossing between  $C_6H_5 \cdot CO \cdot NH \cdot C_6H_4 \cdot N_2^-$  and  $C_6H_5S \cdot CO \cdot NH \cdot C_6H_4 \cdot N_2^-$ , also a relation between  $-SO_3H$  and  $-SeO_3H$ , as predicted by the theory.  $C_6H_5 \cdot CO \cdot C_6H_4 \cdot N_2^-$  and  $C_{10}H_7 \cdot CO \cdot NH \cdot C_6H_4 \cdot N_2^-$  were not related to the first group nor was  $-SO_3H$  to the second. Berger & Erlenmeyer's views (61a) on the relation between molecular size and affinity for antibodies require more and better supporting evidence.

Boyd & Hooker (62) have found that more diazotized atoxyl can be combined with protein than can be accounted for by the tyrosine and histidine content. Other amino acids failed to yield colored compounds under the conditions used, so that the question of how the excess is combined in the protein is left open. The possibility of diazoamino compound formation does not seem to have been considered. Hooker & Boyd (62a) found that atoxylazo gelatin (A) did not precipitate in homologous antiserum which, however, gave precipitates with atoxylazo casein or atoxylazo egg albumin (B). On the basis of inhibition tests it is concluded that A gives rise only to azohistidine antibody, while B forms anti-azotyrosine as well. This hypothesis is supported further by inhibition tests with atoxylazophenol (C) and atoxylazoimidazole, instead of the corresponding tyro-

sine and histidine compounds. Since the latter differ by containing two azo groups instead of one, and C has a *p*-hydroxy group instead of the *o*-hydroxy group in the tyrosine compound, the evidence of the tests loses greatly in force. Finally, the differences between crystalline hen- and duck-egg albumins were studied and explained on the above basis of the existence of antigenic determinants of diverse specificity in a single protein. It is concluded that one of the two antibodies produced by hen-egg albumin is different from that produced by duck-egg albumin, while the other is similar, but not necessarily identical.

That multiple reactive groups determine the outcome of specific precipitation has also received strong support from Heidelberger & Kendall (63), who found that the cross reaction between crystalline egg albumin and antiserum to R-salt-azo-benzidineazo-crystalline egg albumin differed remarkably in its quantitative aspects from the homologous egg albumin-anti egg albumin and dye-antidye reactions. It was concluded that the egg albumin formed a highly dissociated complex with the antidye, and this was shown to be in agreement with Landsteiner's work on azohaptens and specificity. It was also shown that in this particular instance it was not necessary to assume the presence of more than a single antibody in the antidye sera.

Landsteiner & van der Scheer (64) sensitized guinea pigs with azoproteins prepared from *p*-aminosuccinilic and -suberanilic acids and were able to shock them fatally with the homologous bisazo compound prepared by coupling the hapten with resorcinol.

Goebel, Avery & Babers have continued their fundamental study of carbohydrate haptens linked to protein through the diazo reaction. Introduction of an acetyl group into a  $\beta$ -glucoside was found to abolish the cross reactions otherwise observed with unacetylated  $\alpha$ -glucoside (65). The *p*-aminophenol glucosides of the disaccharides cellobiose, maltose, gentiobiose, and lactose were diazotized and coupled with protein (66). The immune reactions of these products appeared in the main to be determined by the glucoside molecule as a whole, the configuration of the terminal hexose molecule, and the position of linkage of the two hexose units in the carbohydrate radical.

*Specific polysaccharides.*—The nature of the discrepancy between the type-specific polysaccharide (SI) of *Pneumococcus* I, as originally isolated, and the antigenic products first reported by Perlzweig & Steffen and later by Schiemann & Casper, Enders, Felton, and Wadsworth & Brown (67) has been cleared up by Avery & Goebel (68), who showed that preparations made without the use of alkali con-

tained 5.9 to 6.9 per cent of acetyl, failed to precipitate at the isoelectric point as did the original SI, protected mice in very small doses against infection by the homologous organism, produced purpura when given in large doses, and removed all protective antibodies from Type I antiserum. Treatment with alkali split off the acetyl groups and gave SI as originally isolated, which was non-antigenic in mice and failed to remove all protective antibody, precipitin, or agglutinin from antiserum. Both products, however, reacted with antiserum at dilutions of one to several millions, and it was this circumstance of equal "titers" which originally led the reviewer to believe that alkali could safely be used in the preparation. The quantitative, absolute method of precipitin determination later developed by the reviewer and Kendall would, however, have made the difference in the alkali-treated material evident at once.

Pappenheimer & Enders (69) also found SI to be a degradation product of an "A" substance which they isolated and which was then found (70) to be identical with the acetyl SI. A similar product appears to have been obtained by Sevag (71) after repeatedly freezing aqueous *Pneumococcus*-I suspensions in liquid air and removing protein by shaking with chloroform and amyl alcohol.<sup>5</sup> The same method was used to isolate a polysaccharide from egg white to which immunological activity is rather incautiously ascribed, especially in view of Ferry & Levy's negative findings (72).

Francis (73) has found both the acetyl and deacetylated SI to be antigenic when given intradermally in man. Oram (74) has shown differences between SI and pneumococcus "leucocidin."

Lancefield (75) has found that hemolytic streptococci of animal origin fall into a number of groups characterized by group-specific polysaccharides which differ from that of group A, the human pathogens. In the B, or bovine group, type-specific carbohydrates were also encountered. Julianelle & Wieghard (76) have similarly found that human pathogenic strains (Type A) of *Staphylococcus* possess a specific polysaccharide chemically distinct from that of the Type B, avirulent, non-pathogenic strains. Linton & Shrivastava (77) have isolated, among the hydrolysis products of the specific polysaccharides of agar-grown cholera vibrios, a non-reducing fraction, glucuronogalactose, and galactose from some strains and arabinose from others.

Sordelli, Deulofeu & Ferrari (78) have found that the antigen in

<sup>5</sup>  $[\alpha]_D$  of the product is erroneously given as 21.9° instead of 217°.

agar-grown *B. anthraci*, which produces antibodies which precipitate agar, may be easily dissociated by washing the organisms. Zozaya & Medina (79) have shown their previously reported cross reactions of specific polysaccharides to be partially due to agar. Pneumococcus "C" substance absorbed agar antibodies from serum, indicating a chemical relationship between the polysaccharides.

The partial hydrolytic products of the specific polysaccharide of Type III pneumococcus, with the exception of the ultimate aldobionic acid unit, were found by Heidelberger & Kendall (80) to precipitate homologous horse antiserum, but not rabbit antisera. Reaction nevertheless occurred in the latter case, as higher concentrations inhibited specific precipitation of S III. A somewhat similar weakening in precipitating power toward rabbit sera, but not horse sera, was found by Levine & Frisch (81) in phage-inhibiting extracts of *B. Aertrycke* heated with dilute acid at 80°. At the same time the intensity of the phage-inhibiting action was greatly increased, suggesting the unmasking of specifically reactive groups, as had been found by Heidelberger, Avery & Goebel (82) in the partial hydrolysis of gum arabic. Munday & Seibert (83) have called attention to the higher values for reducing sugars obtained in tubercle-bacillus-polysaccharide hydrolysates with the Hagedorn-Jensen method than with the Shaffer-Hartmann method, ascribing the difference largely to the pentose present. The errors introduced by the presence of products of protein hydrolysis were also evaluated. Hydrolytic enzymes for various specific polysaccharides have again been encountered (84).

*Lipoids and miscellaneous substances.*—Evidence is given by Jorpes & Norlin (85) that of the blood-group specific substances A, originally thought to be lipoids, then carbohydrates, the actual hemagglutinin contains relatively intact proteins, occurring in the "alloy-protein" fraction of urine and separable from the polysaccharide sheep-hemolysin-inhibiting factor by precipitation with tannin. Their activity was destroyed by proteolytic enzymes. The polysaccharide factor was found by Freudenberg & Eichel (85a) to contain galactose, amino sugar, and acetyl. Misawa (86) and Landsteiner & Jacobs (87) have shown that adsorption of purified Forssman hapten on various substances does not convert the hapten into an antigen, as it does the crude extract used by Gonzales and Armangué.

Rudy (88) has found that the so-called lipid hapten of the brain becomes more and more water soluble as it is purified, giving reactions for acid, nitrogen, and sugar. He has also reviewed the subject of

lipoid haptens (89). Wadsworth, Maltaner & Maltaner (90) have found that highly purified cephalin or lecithin react neither as antigens nor haptens when the authors' quantitative complement-fixation method is used, involving adequate controls and proper consideration of the anticomplementary action, especially of cephalin when exposed to air. Maltaner & Maltaner have also found that union of cephalin with serum proteins to form water-soluble complexes did not change the protein specificity or convert cephalin to a hapten. Cholesterol-swine-serum mixtures yielded antisera which showed only non-specific fixation of complement, as did normal serum-cholesterol mixtures.

On the other hand, tubercle-bacillus lipoids, particularly the unusually constituted phosphatides, appear to be definitely antigenic (34, 91), and Tropp & Baserga (92) have isolated a spleen polydiaminophosphatide, which, in contradistinction to cerebrin and lignoceryl-sphingosin, gave rise to complement-fixing antibodies when injected with pig serum. Seibert, Long & Morley (92a) found S-tubercle-bacillus strains to contain more lipoid than R strains. Hettche (92b) has found the bactericidal and hemolytic action of *B. pyocyaneum* lipoids to be due to the liquid fatty acids. The bactericidal action of fatty acids of known constitution was also studied.

Wedum (93) was unable to observe any evidence of immunological activity on injection into guinea pigs of a number of synthetic glucosides ranging in molecular weight from 222 to 1147.

#### ANTIBODIES AND COMPLEMENT

Buttle (94) has reported ingenious experiments which failed to locate the source of production of diphtheria antitoxin in rabbits. McMaster & Hudack (95) obtained evidence of the formation of agglutinins in the lymph nodes of mice. References to the influence of diet and various substances, or treatments, on antibody formation are grouped under reference 96.

The nature of antibodies and the mode of their formation were discussed by Eastwood (97) with the aid of a stereochemical analogy but with gloomy distrust of the chemical approach to an understanding of immunity. Mudd (98) has put forward a theory of antibody formation much like that of Breinl & Haurowitz,<sup>6</sup> but more specific, and based on the analogy to the temporary union of an enzyme with active groups in a polypeptide. Baldassi (99) observed that develop-

<sup>6</sup> Cf. Heidelberger, M., *Ann. Rev. Biochem.*, 1, 664 (1932).



ment of diphtheria antitoxin in horses was marked by an increase in the optical rotation of the serum, in some cases even before the appearance of antitoxin.

Studies on the purification of antibodies again point to their protein nature. Silber & Demidowa (100) were unable to reduce the protein content of typhoid agglutinins below 0.16 per cent; on heating, agglutinin disappearance and protein denaturation ran parallel. The resemblance of the heat destruction of tetanus antitoxin to protein denaturation was pointed out by Gerlough & White (101). Felton & Kauffmann (102) have given additional data on highly purified pneumococcus antibody; dissociated from combination with specific polysaccharide it showed minimum solubility at pH 6.8, differing from the pseudoglobulin associated with it in serum only by its relative insolubility in water and its basicity. The antibody protein was digested by pepsin and trypsin with loss of its immunological properties. The dissociated precipitin gave positive tests for agglutinin, precipitin, bacteriolysin, opsonin, complement fixation, and protection, supporting the unitarian theory. Girard & Lourau (103) have also observed that agglutinins and hemolysins differ from other serum proteins in their higher isoelectric points as shown by cataphoresis. Kirk & Sumner (104) have effected a high degree of purification of antiurease by specific precipitation with urease, dissociation of the enzymatically active precipitate with 0.05 *N* HCl, which also destroys the urease, neutralization to pH 5, and removal of the denatured enzyme. Pepsin and papain digest the purified antibody. Its combining relationships with urease were studied, but the data, on careful analysis, lead to opposite conclusions than those drawn. Ramon (105), having separated antitoxin specifically from the accompanying non-specific pseudoglobulin, has ignored Marrack & Smith's careful work in concluding that antitoxin is possibly non-protein.

References on the distribution of antibodies and antitoxins in the protein fractions of the sera of various animals, and the failure of serum lipoids to affect either distribution or reactivity are grouped under 106; the last reference deals with the separation of serum lipase from antitoxin.

An absolute, quantitative method for the determination of agglutinins has been proposed by Heidelberger & Kabat (107). As in the precipitin method from the same laboratory, the amount of antibody nitrogen precipitated by an excess of antigen (in this case a measured amount of bacterial suspension) is measured, deducting the nitrogen

in the bacteria used. In this way it is possible to determine agglutinin in mg. per cc. with a high degree of accuracy instead of recording a vague "titer." The method has as yet been perfected only for pneumococcus S [Dawson "M" (108)] and R (Dawson "S") strains. With the method it has been possible to show an exact quantitative correspondence between anticarbohydrate as precipitin and agglutinin. Heidelberger, Kendall & Soo Hoo (109) have studied antibody formation to the red dye, R-salt-azobenzidineazo-crystalline egg albumin with the aid of the absolute quantitative method, finding that as little as 0.55 mg. of the antigen, given in a series of minute doses, may stimulate the production of more than 200 times its weight of precipitin. With larger amounts of antigen relatively less antibody was formed, but the sera were of higher antibody content. The variation of antibody content in different rabbits was followed through a number of courses of injections, as was the variation in individual sera over long periods of storage. The same red hapten has been used by Marrack (110) as an aid in tracing the behavior of agglutinins. Vincent (111), in elaborating his ideas on toxin formation, has quoted Bourdin as calculating that a horse may produce enough antitoxin to neutralize more than 1,000 times as many  $L_0$  doses of toxin as were used to stimulate the antitoxin production—another blow to the theory that antigen fragments are contained in antibody. Brown (112) has found excellent correspondence, in most cases, between precipitation of homologous antisera and antibody solutions with "cellular," or better, acetyl polysaccharide of Type I pneumococcus and the mouse-protection values, confirming earlier work on antisera in which the deacetylated SI had been used.<sup>7</sup>

Grützner (113) has shown that old antiricin sera could be restored by treatment with 0.05 N to 0.01 N NaOH, and has purified antiricin by extracting dried sera in this way. In some instances the activity of fresh sera was increased, leading to belief in a pre-stage of antibody formation requiring activation. Mudd *et al.* (114) have found that frozen sera, evaporated in a high vacuum, form fluffy masses easily soluble in water and retaining their activity well.

Hooker & Boyd (115) have discussed the relation of antibody to antigen and have shown, as did Berger & Erlenmeyer, that antisera to atoxylazoprotein contain no more arsenic than normal sera. It was also shown that it was necessary for atoxylazocasein to contain at least

<sup>7</sup> Cf. Heidelberger, M., *Ann. Rev. Biochem.*, 2, 503 (1933).

thirteen arsenic azo groups in order to precipitate with anti-atoxylazoe-egg white, but calculations of the number of anti-hapten groups per molecule of antibody are less convincing.

The specificity changes undergone by antibodies when iodinated, formalinized, racemized with sodium hydroxide, and coupled with diazotized aromatic amines have been studied by Breinl & Haurowitz (116). With the last reagent agglutinins and species specificity were destroyed to about the same extent, while iodination affected the former more strongly. The findings were considered consistent with the hypothesis that antibodies are proteins, and that, just as in the case of antigens, aromatic groupings are important in determining their specificity. Mudd & Joffe (117) found that formalin treatment shifted the isoelectric point of agglutinins toward the acid side and reduced the end titer.

Lumsden & Macrae (117a) have reported antibodies to cancer cells.

Silber & Schafran (118) have called attention to a number of properties of complement which vary in the same direction as the protein denaturation taking place. Gordon & Thompson (119) studied the effect of salts on complement activity, and concluded that this activity is associated with a particular state of aggregation of serum proteins. Bancroft, Quick & Stanley-Brown (120) have given evidence that complement and prothrombin are not identical, while Maltaner & Maltaner (121) have shown that the inhibition of complement by cephalin parallels the activity of cephalin in the process of coagulation and may be reversed by calcium chloride.

#### THE CHEMISTRY OF IMMUNE REACTIONS

*General.*—Moriyama (122) has attempted a colloidal interpretation of the mechanism of immune reactions. According to Fuchs (123) the "residual nitrogen" diminishes in serum when antigen and antibody combine, the decrease being represented by a change from, for example, 0.38396 mg. per cc. to 0.36254 mg.! Since the effect is very slight at best, and purified antigen and antibody are known to combine in the absence of "residual nitrogen," the reviewer is skeptical of this and subsequent work from the same laboratory on tumor antibodies.

*Agglutination.*—Ivánovics (124) has used a "quantitative" method, accurate to  $\pm 10$  to 15 per cent, and concludes that the binding of agglutinin to dysentery bacteria is a physical process, not chemical.

Duncan (125) and Miles (126) have discussed the factors influencing agglutination in optimal proportions and have studied and explained the great difference in the optimum depending on whether antigen or antibody is diluted. In the former case, corresponding to the Dean-Webb procedure, true antigen-antibody equivalence is more nearly represented by the optimum. An absolute method for agglutinin determination, conforming to the criteria of quantitative chemical analysis, has been referred to already (107).

Jones & Little (127) have studied the increase in volume occurring when bacteria are agglutinated by immune serum and find it to be greater than can be accounted for by the amount of protein absorbed. Olitzki (128) has discussed the electric charge of bacteria sensitized with purified agglutinins, but has failed to realize that precisely these effects would be expected if antibodies were proteins. Sédallian & Clavel (129) have recovered up to 50 per cent of agglutinin and protective antibodies by treating agglutinated streptococci with dilute hydrochloric acid.

*The precipitin reaction.*—Frequent reference to this reaction has already been made. An absolute, quantitative method for the measurement of precipitins is given by Heidelberger *et al.* (109). Culbertson (130) has given the details of his modification (accurate to about 10 per cent) of this method and has applied it to the determination of blood volume and the measurement of circulating antibody before and after the injection of egg albumin. It is concluded that circulating antibody accounts for the egg albumin which disappears, and that cellular antibodies are not immediately available (131). The influence of pH on the egg albumin-anti egg albumin reaction was also taken up (132). Duncan has studied the optimal proportions method in the precipitin reaction much as in the case of agglutination (133). Taylor (134) has also applied the method to the egg albumin-anti egg albumin system, but the cumbersome technique and discussion of the difficulties do not inspire confidence in its use for precise work. With Adair & Adair (135) he has checked the method by nitrogen analyses, finding that the Dean-Webb optimum corresponds closely to the equivalence point. Maximum precipitation (total nitrogen) occurred when 1.6 to 2.4 times the optimal amount of egg albumin was present. There was no inhibition with very high concentrations of antibody. The weights of the precipitates agreed closely with those calculated from the nitrogen content, confirming the protein nature of the precipitate. Jones (136) has proposed measurement of the volume of

the precipitate as an accurate titration of precipitin, but its accuracy is doubtful since it indicates a linear relation between the amount of antigen added and the amount of precipitate, up to and somewhat beyond the equivalence point. In inhibition tests with atoxylazoprotein-antibody, Haurowitz & Breinl (137) obtained positive results with arsanilic acid but not with the corresponding stibonic acid as should have followed from Berger & Erlenmeyer's theory of similar force fields. Lumière & Meyer (138) have studied the albumin and globulin content, viscosity, surface tension, colloid osmotic pressure, and volume of the proteins in the supernatant fluid, concluding that the reaction is purely physical and that the molecules in the supernatant fluid have a greater volume than in normal sera owing to increased hydration. The results, however, are easily explained on a chemical basis, particularly as the proportions of antigen and antibody used were such as to throw the reaction partly into the inhibition zone, where large molecules of soluble antigen-antibody compound would be present. Following the demonstration by Heidelberger & Kendall that the high antibody-hapten ratios observed in the precipitin reaction between Type-III-pneumococcus specific polysaccharide and antibody are due to the low molecular weight of the polysaccharide, Hooker & Boyd (139) have made an interesting comparative study of the equivalence point ratios of other systems as well, including egg albumin, hemoglobin, pseudoglobulin, and hemocyanin, with molecular weights ranging from 4,000 to 2,000,000. With certain assumptions a mathematical expression was derived from which equivalence point ratios corresponding to given molecular weights were calculated. In some instances remarkably close agreement was obtained with the observed values. While there is undoubtedly a relation between the antigen-antibody ratios observed in the precipitin reaction and the relative molecular weights of the reactants, there is still uncertainty regarding almost every factor involved in the quantitative formulation of such a relation. Hooker & Boyd point out that a typical specific polysaccharide may contain 500 times as many molecules per unit weight as, for example, hemocyanin, thus accounting for the high limiting dilutions observed in precipitin reactions involving the carbohydrates and the relatively low limiting titers of protein-antiprotein systems. The same workers (140) have also used the precipitin reaction to bring forward very definite evidence that serum albumin to which heparin had been added was still albumin, and had not been converted to globulin as claimed by A. Fischer. The precipitin reaction has been

followed in pneumonia by Viktorow & Masel (141), who emphasize the necessity of controlling pH and salt content in urines set up with antibody. Beale (142) has used the precipitin reaction for the measurement of plant viruses. An interesting development in virus diseases was Hughes' discovery (143) that the serum of monkeys acutely ill with yellow fever contained a precipitinogen in the albumin fraction which was entirely independent of the virus, and was possibly formed by the action of the virus on the body proteins. This substance induced precipitin formation in the sera of recovered cases in man and monkeys. An apparently similar observation in trypanosomiasis has been made by Poindexter (144).

*Anaphylaxis and allergy.*—Jones & Fleischer (145) have found the pseudoglobulin of normal horse serum or diphtheria antitoxin to be more active in causing serum sickness in rabbits than the euglobulin or albumin, the activity possibly being due to some other substance precipitated with the pseudoglobulin, or to variations in the configuration of this fraction in individual sera. Davidsohn (146) believed the Forssman antigen in the injected horse serum to be the responsible factor. Rentz & Kaktin (147) reported that daily peroral administration of acid to guinea pigs increased the severity of anaphylactic shock about 33 per cent, while alkali administration diminished it about 50 per cent. Paul & Popper (148) have shown that in dogs and guinea pigs, but not in rabbits, complement is diminished in histamine shock as well as in anaphylactic shock. Landsteiner & Jacobs (149) were able to induce a specific skin sensitivity in a number of guinea pigs by repeated injections of salvarsan,  $p\text{-C}_6\text{H}_4(\text{NH}_2)_2$ ,  $p\text{-ONC}_6\text{H}_4\text{N}(\text{CH}_3)_2$ ,  $1,2,4\text{-C}_6\text{H}_3\text{Cl}(\text{NO}_2)_2$ , and suberanic acid azoresorcinol.

*Hemolysis and complement fixation.*—Diacono (150) has contributed a review and reported the recovery of about 80 per cent of the anti-sheep-cell hemolysin in guinea pig antiserum; it was present in the globulin fraction precipitable by carbon dioxide. Serum titers were influenced by the animals' diet. Cholesterol *in vivo* did not influence the reaction; *in vitro*, it inhibited the alexin only. Györfy (151) has dealt with inhibiting effects in beef-serum systems. Sierakowski & Zablocki (152) found that the pH-velocity curve of hemolysis greatly resembled that of tryptic action, with an optimum from pH 7.6 to 8.0, in line with the finding of Maltaner & Maltaner (153) that the reversible inhibition of hemolysis or clotting after adsorption with magnesium hydroxide is due to the increased alkalinity. Randall

(154) has traced the inhibiting effect of sodium cyanide to its action on complement, while Klopstock & Neter (155) have studied the inhibiting action of tannin.

Hambleton (156) has discussed complement fixation as a secondary effect due to the acquisition of appropriate surface properties by suitably modified antigens.

*Miscellaneous reactions.*—Ward & Enders (157) found that antibody to pneumococcus specific polysaccharide appeared to be the only phagocytosis-promoting factor in these immune sera. If sufficient time was allowed, the same endpoint was reached, with or without complement.

Alloway (158) has studied the substance responsible for the change of R pneumococci (Dawson "S") into S forms (Dawson "M").

Studies on isohemagglutination have been reported by Ottensooser & Lenzinger (159), and the water-soluble group-specific substances have been further purified by Hallauer [(160), cf. also (85)].



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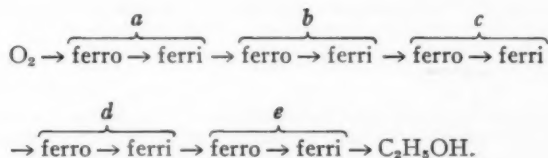
## THE CHEMISTRY OF BACTERIA\*

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### RESPIRATION

*Bact. pasteurianum* and *Acetobacter* have furnished material for the spectroscopic study of the *Atmungsferment* system. According to Warburg's scheme the iron catalysis of *Bact. pasteurianum* may be represented thus:



*a* represents the oxygen transporting enzyme; *c*, *d*, and *e* are the three components of cytochrome; *b* is an iron compound which reduces *a* and oxidises *c*. The ferro-form of *a* alone is autoxidisable, and, as the oxidation proceeds more rapidly than the reduction, the ferri-form alone is present in respiring cells. The ferro-form of *a* reacts with carbon monoxide, and the residual respiration then becomes dependent on oxygen pressure. The action of cyanide is independent of oxygen pressure, i.e., it inhibits the reduction of the enzyme. Hence in the search for the bands of the oxygen transporting enzyme one must expect a system which is shifted by carbon monoxide and which disappears on saturation with oxygen or on treatment with cyanide. Cytochrome fulfills none of these conditions.

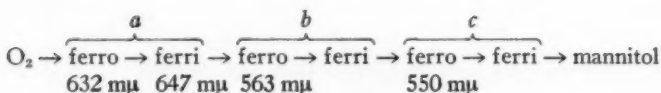
*Bact. pasteurianum* has an unusually high  $Q_{O_2}$ ; in the presence of ethyl alcohol and at 25° it amounts to 1,000. Anaerobically, in the presence of ethyl alcohol, three cytochrome bands are visible at 550, 553, and 563 mμ (the band at 603 to 605 mμ is absent). On saturation with oxygen these disappear, and cyanide and carbon monoxide inhibit the oxidation. In very thick cell suspensions an-

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other band is seen at 589  $m\mu$  which disappears on oxidation, but is sharpened by cyanide; if treated with cyanide and shaken with oxygen it disappears (while the cytochrome bands are unchanged). On saturation with carbon monoxide, the band moves to 593  $m\mu$ . This band fulfills all the conditions expected of the oxidising enzyme (Warburg, Negelein & Haas). Keilin doubts whether this band is that of the oxidising enzyme, but considers that it is probably due to changes in one of the cytochrome components. In reply, the original authors point out that Keilin's suggestion is unacceptable, because cytochrome constituents are neither autoxidisable nor affected by cyanide and carbon monoxide, whereas the band at 589  $m\mu$  fulfills all three of these conditions (Warburg & Negelein).

In the case of *Azotobacter* the oxidising system is represented as follows:



Oxygen oxidises ferro-*a* to ferri-*a* (632  $\rightarrow$  647  $m\mu$ ); carbon monoxide reacts with ferro-*a* (632  $\rightarrow$  637  $m\mu$ ); cyanide reacts with ferri-*a*, causing the band at 647  $m\mu$  to disappear, but no new band occurs corresponding to a ferri-CN compound. Neither oxygen, carbon monoxide, nor cyanide reacts with iron compounds *b* and *c* (cytochrome components), so if ferro-*a* is blocked with carbon monoxide or ferri-*a* with cyanide, ferri-*b* and -*c* can still be reduced, but ferro-*b* and -*c* cannot be re-oxidised. This system has several points of difference from that of the acetic bacteria: (*a*) on treatment with cyanide the band at 647  $m\mu$  disappears and no cyanide band appears in its place (this corresponds with the behaviour of haemin, but contrasts with *Bact. pasteurianum*, where after treatment with cyanide a band is present in the red); (*b*) the position of the  $\alpha$ -band of ferro-*a* in acetic bacteria is in the yellow at 589  $m\mu$ , and in *Azotobacter* in the red at 632  $m\mu$  (Negelein & Gerischer).

A discussion on the relative importance, as oxygen carriers, of the *Atmungsferment* system and the yellow pigment in aerobic and anaerobic cells where both occur together is significant in bacterial respiration (Warburg). By estimating the amount of pigment in the cell and the velocity of the reaction,  $\text{O}_2 + \text{FH}_2 = \text{H}_2\text{O}_2 + \text{F}$  (where

F = the pigment), together with the total oxygen uptake of the cell measured manometrically, and that part of it which goes through the cytochrome system measured photo-electrically, it was found that in the case of baker's yeast and *Acetobacter* the cytochrome system is responsible for practically all the respiration, the yellow pigment only effecting 0.5 and 0.1 per cent, respectively, of the oxygen uptake. In certain lactic acid bacteria, on the other hand, the iron system is completely absent, and 100 per cent of the respiration occurs through the yellow pigment; but, as these bacteria live largely anaerobically and rely very slightly on molecular oxygen for energy, this leaves us at present without an important rôle for the yellow pigment.

Frei, Reidmüller & Almasy have made an extended study on the distribution among forty-one species of bacteria of the constituents of the cytochrome spectrum: indophenol oxidase, peroxidase, catalase, and haematin. As a result, the bacteria examined were divided into four groups:

Group 1. Both cytochrome and indophenol oxidase are present; with one exception they have a complete cytochrome spectrum, although some facultative anaerobes (e.g., *B. pyocyaneus*) are included in the group; all contain catalase and peroxidase.

Group 2. Cytochrome (some components) is present, but not indophenol oxidase.

Group 3. Indophenol oxidase is present, but not cytochrome.

Group 4. Neither cytochrome nor indophenol oxidase is present.

Groups 1, 2, 3, and the facultative anaerobes of group 4 usually contain peroxidase and catalase. It is noteworthy that organisms may belong to different groups at different ages. The cytochrome spectra of forty-four species of bacteria and seventeen yeasts have been mapped (Tamiya & Yamaguchi).

Yamaguchi has made a study classifying bacterial respiration as follows:

Group 1. Respiration unaffected by cyanide or carbon monoxide.

Group 2. Respiration inhibited by cyanide but not by carbon monoxide.

Group 3. Respiration inhibited by cyanide and carbon monoxide, but the latter inhibition not reversed by light.

Group 4. Respiration inhibited by cyanide and carbon monoxide, and the latter inhibition reversed by light.

Several interesting studies on oxidation-reduction potentials are at hand. In the case of *M. denitrificans* (Elema, Kluyver & van

Dalfsen), the potential appears to be closely linked with denitrification. Washed suspensions of the organism were used in buffered media with nitrate (or nitrite) and ethyl alcohol as the hydrogen donor. Starting at Eh 0.1 the potential fell to  $-0.15$  to  $-0.20$  volt, where it remained so long as any nitrite was present; it then fell abruptly. It was shown that the fall in potential was governed by the simultaneous presence of nitrite and bacteria. To a suspension of bacteria in which the nitrite was exhausted and the potential had fallen to  $-0.236$  volt, an addition of a small amount of nitrite ( $86 \gamma$   $\text{NaNO}_2$  in 15 cc.) caused an immediate return to its original level, followed by a further fall as the nitrite became exhausted. The potential was lowered by the addition of cyanide. These facts led to the conclusion that the reactions governing the potential are the reduction of the nitrite to nitrogen and water and the oxidation of the hydrogen donor. The action of cyanide ( $1 \gamma$  KCN per cc.) resulted in a lowering of potential and an inhibition of the evolution of nitrogen, although nitrite continued to disappear. This suggested that cyanide had blocked the reaction at the stage of hyponitrite, which was confirmed by the appearance of nitrous oxide in the gas phase.

Phthiocol (2-methyl-3-hydroxy-1,4-naphthoquinone), the pigment of the human tubercle bacillus, has been shown to be the oxidant of a reversible system with a low potential: Eh at  $30^\circ$  and pH 7.3 and 52.9 per cent oxidation is  $-0.207$  volt (Ball).

The potential of the yellow oxidation pigment has also been determined; Eh at  $20^\circ$  and pH 7.21 is  $-0.215$  volt (Bierich & Lang).

The hydrogenase system (*Bact. coli*) has now been shown to be reversible (Green & Stickland). Using methyl viologen as indicator, it was shown that for any given pH and partial pressure of hydrogen the degree of reduction of the dye was the same whether colloidal palladium or *Bact. coli* were used as catalysts. Thus *Bact. coli* catalyses the reaction,  $\text{H}_2 \rightleftharpoons 2\text{H}^+ + 2\text{e}$ , reversibly; the system is the most negative so far observed in biological systems; for example, Eh =  $-0.401$  volt at  $30^\circ$ , pH 7.02, and hydrogen pressure of 600 mm.

Messing has followed the potential change of nutrient broth with and without sugar and sulphhydryl compounds, respectively. He finds that, when these substances are added without bacteria, no important fall in potential occurs, while in the presence of bacteria a rapid fall of about 300 mv. results. He therefore deduces that these

substances do not exert their effect on bacterial growth by causing a fall in potential favourable to cell-multiplication, but possibly by favouring some reaction between the cell and the medium, as a result of which the potential falls.

A re-examination of the relationship between fermentation and respiration on the lines of Meyerhof & Finkle has now been undertaken [Davis (1)]. *Bact. delbrückii* has no catalase, and forms hydrogen peroxide aerobically; hence on sugar it has an R.Q. of 0.5. Methylene blue and *p*-phenylenediamine raise the oxygen uptake 100 to 300 per cent, and decrease the formation of hydrogen peroxide by 25 per cent, resulting in an R.Q. of 0.7 to 0.9. Iodoacetic acid inhibits the oxidation of glucose but not of lactic acid, showing that glycolysis precedes oxidation. Pyruvic acid is more rapidly oxidised than lactic acid, due to the secondary oxidation of the former by hydrogen peroxide. The original work of Meyerhof & Finkle did not take into account the action of hydrogen peroxide, hence the necessity for redetermining the Meyerhof- and oxidation-quotients.

	Meyerhof Quotient*	Oxidation Quotient†
Old values.....	2.2	6.6
New values.....	1.0	3.0

\* Mols lactic acid oxidised anaerobically minus mols lactic acid oxidised aerobically divided by mols oxygen.

† Mols lactic acid spared divided by mols lactic acid oxidised.

The other organism studied (*Bact. cereale*) behaves as an aerobe with a typical haemin-containing respiratory system: does not produce hydrogen peroxide, grows well in air, and has a respiration unaffected by methylene blue and inhibited by cyanide and carbon monoxide. This organism has an oxidative fermentation, giving in bicarbonate buffer an excess of carbon dioxide over oxygen (R.Q. > 1).



$$\text{CO}_2/\text{O}_2 = 2.$$

Anaerobically, very little extra carbon dioxide and no pyruvic acid are formed; with fluoride, extra carbon dioxide and pyruvic acid

are equally inhibited. The final products of fermentation are ethyl alcohol, acetic acid, and acetoin.

In calculating the oxidation quotient, allowances must be made for the extra carbon dioxide due to aërobic decomposition of pyruvic acid and the carbon dioxide liberated by the acetic acid; the Meyerhof quotient then amounts to 1.6 [Davis (1)].

*Lactobacillus casei* differs in having an almost negligible oxygen uptake and in forming no hydrogen peroxide; its respiration is not raised by methylene blue, which merely inhibits glycolysis; the  $Q_{O_2}$  is very low (2.6 to 0) [Davis (2)].

The  $Q_{O_2}$  and glycolysis quotient in oxygen ( $Q_{O_2^{O_2}}$ ) and in nitrogen ( $Q_{O_2^{N_2}}$ ) for a number of pathogenic organisms have been determined;  $Q_{O_2}$  varied according to the medium on which the organisms had been grown, while  $Q_G$  suffered from the fact that in many cases the ratio between acid formed and sugar decomposed was not known (Fujita & Kodama).

Sevag, in a study of the respiration of the pneumococcus, notes that the hydrogen peroxide produced aërobically on sugar reacts with the pyruvic acid formed, which thus, like added catalase, protects the enzymes from destruction. Virulence is similarly destroyed by hydrogen peroxide and restored by catalase and pyruvic acid. The observations are made that endogenous respiration is a measure of the integrity of the cell and that gram-negative cells lack enzymic activity. Some chemical differences are noted between virulent and avirulent types; for instance, oxidation of ethyl and propyl alcohol is said to be effected only by the avirulent types.

The production of hydrogen peroxide during the aërobic fermentation of glucose by *Lactobacillus bulgaricus* is reported (Fromageot & Roux).

Work on the production of a green pigment from laked blood, and also from oxy-, meth-, and carboxy-haemoglobin, by certain streptococci and pneumococci has been further studied (Anderson & Hart). It is now found that the action of the bacteria can be imitated by reducing systems, such as ascorbic acid, cysteine (not very active), cysteine with glucose, glycine with glucose, and glycine with dihydroxyacetone; glucose, glycine, and dihydroxyacetone were inactive when used separately. Washed suspensions of bacteria: those giving the green colour on blood-agar plates as well as others (*Str. faecalis*, *Bact. coli*, *Staph. aureus*), produced the pigment in the presence of glucose, and glucose with glycine. The production of

the pigment appears to be a complicated process involving a reduction of oxyhaemoglobin to reduced haemoglobin, followed by an oxidation; the reducing system is supplied by the bacterial dehydrogenases. The fact that certain organisms can produce the pigment, in specially controlled conditions, yet fail to show the well-known green colour on blood-agar plates is attributed to acid reaction, unsuitable oxidation-reduction potentials, and, in some cases, to the decrease in haemoglobin content due to haemolysis.

The identity of the pigment has not been determined, but a close resemblance to "green haemin" has been pointed out.

It is noted by Gordon that certain streptococci are irregular in the production of green colour on heated blood agar, and the effect is increased by adding sodium lactate. This is attributed to the action of the latter as a hydrogen donator resulting in greater production of hydrogen peroxide. It may also be connected with the greater power of reducing haemoglobin and consequent increase in the green pigment.

#### ANAEROBIC MECHANISMS

It has already been shown that the intact cells of many facultative anaerobes contain enzyme systems in virtue of which one substance can be reduced and another oxidised; for example, formate reduces nitrate, the enzymes concerned being formic dehydrogenase and nitratase; lactate reduces fumarate, the enzymes being lactic dehydrogenase and succinoxidase, and it is inferred that upon such reactions the anaerobic cell depends for energy. Schott & Borsook showed that, whereas intact cells of *Bact. coli* could (for example) reduce pyruvate by formate, the toluene-treated organism could not do so, though the power was restored by a trace of some oxidation-reduction indicator. Green, Stickland & Tarr have now extended this observation to a number of oxidation-reduction systems, bacterial and otherwise. They have shown, for example, that a cell-free preparation from *Bact. coli*, containing both formic dehydrogenase and nitratase, no longer reduces nitrate by formate, though it does so in the presence of a small amount of oxidation-reduction indicator. The latter obviously functions by being reduced by one system and oxidised by the other, and one must therefore be chosen whose equilibrium point lies between the range of the two enzyme systems. This is exemplified in the following table:

Negative System	Indicator	Positive System	pH	Time, Hours	Product Estimated	Concentration of Product M
Formic dehydrogenase (from <i>Bact. coli</i> ) + formate	Ethyl capri blue	Nitratase (from <i>Bact. coli</i> ) + nitrate	7.0	3	Nitrite	$1 \times 10^{-3}$
Lactic dehydrogenase (from <i>Bact. coli</i> ) + lactate	Ethyl capri blue	Nitratase (from <i>Bact. coli</i> ) + nitrate	7.0	3	Nitrite	$5 \times 10^{-3}$
Xanthine oxidase (from whey) + hypoxanthine	Benzyl viologen	Lactic dehydrogenase (from <i>Bact. coli</i> ) + pyruvate	7.0	5	Uric acid	$5 \times 10^{-3}$

As a rule the indicator carriers work at very low concentrations, e.g., Nile blue and capri blue at  $3 \times 10^{-7} M$ , and for each reaction the maximum velocity is obtained at some point on the potential scale between the potentials of the two enzymes. Thus the efficiency of any indicator for any reaction depends on its position in the scale.

From these observations the authors advance the theory that there exist in the intact cell, substances which, like the indicators *in vitro*, link together the various oxidations and reductions. Attempts to extract such substances have so far been unsuccessful, the only naturally occurring substance functioning in this way being pyocyanine. Substances which have been tried and found to be inactive are cytochrome, Warburg's yellow pigment, glutathione, ascorbic acid, lactoflavin, and ovoflavin.

A step forward in our knowledge of the metabolism of the strict anaerobes appears in a study of *Cl. sporogenes* (Stickland). Owing to the impossibility of cultivating members of this group on simple synthetic media, the mechanisms by which they obtain energy have so far remained hidden.

Moreover, it appears from this study that, using dyestuffs as hydrogen acceptors, the organism is unable to oxidise such common donors as formate, lactate, succinate, and glucose. As protein digests form the usual medium for growth, the power of the organism to oxidise amino acids was tried, and it was found that *D*-alanine, *L*-leucine, *D*-valine, and *L*-phenylalanine all served.

The search for hydrogen acceptors showed that substances (nitrate and fumarate) which are active with facultative anaerobes, are with-



out action here; on the other hand, using a negative dye as indicator (benzyl viologen), glycine, proline, and hydroxyproline were found to function.

It was subsequently shown that any one of the hydrogen donors, in the presence of cell suspensions, reacted with any one of the hydrogen acceptors. The device for showing this depends on the observation that during the oxidation of the donator the nitrogen is quantitatively liberated as ammonia, the same being true for the reduction of the acceptor (with the exception of proline and hydroxyproline), while in the presence of either donator or acceptor, separately, no deamination occurs. Hence, liberation of ammonia can be used as a measure of reciprocal oxidation and reduction. Using this technique each donator was found to react (in the presence of the bacterial suspension) with each acceptor, and *l*-aspartic acid and *d*-glutamic acid were added to the list of hydrogen donors and *l*-histidine and *l*-tryptophane to the list of acceptors. Serine, and to a less extent tyrosine, though acting neither as hydrogen donors nor acceptors, were deaminated by the organism alone.

The course of the interactions shown to occur is not yet determined, but preliminary results go to show that *d*-alanine and glycine react thus:  $\text{CH}_3 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH} + 2 \text{CH}_2(\text{NH}_2) \cdot \text{COOH} + 2 \text{H}_2\text{O} = 3 \text{CH}_3 \cdot \text{COOH} + 3 \text{NH}_3 + \text{CO}_2$ .

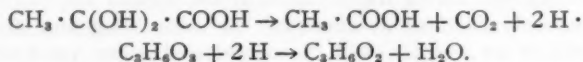
#### FERMENTATION

New and interesting facts have been brought to light on propionic acid fermentation by *Propionibacterium arabinosum* [Wood & Werkman (1)]. During the fermentation of glucose in yeast water, it was found that up to, and at the moment of, the disappearance of the hexose the total fermentation products did not account for the sugar fermented; by the end of the fermentation this discrepancy disappeared, and 99 per cent of the sugar was accounted for. This indicates the intermediate formation of a non-reducing substance which is subsequently fermented. The substance may be identical with the "slime" noted when the fermentation is attempted in too alkaline a medium. As it occurs with other varieties of propionic bacteria, it is possibly an essential step in fermentation, and may account for the formation of succinic acid from 3-carbon compounds noted by earlier workers.

In the same fermentation, a non-volatile ether-soluble compound containing a carbonyl group was isolated; the identity of this sub-

stance was not determined, but it was stated to be neither pyruvic acid nor methyl glyoxal.

In a subsequent paper [Wood & Werkman (2)] the authors isolated pyruvic acid in the fermentation of glucose, using sulphite as a fixative. This confirms van Niel's scheme (1928) of propionic acid fermentation in which pyruvic acid holds a key position.



In a similar manner propionaldehyde was isolated by sulphite in the fermentation of glycerol, though it has not so far been obtained from hexose [Wood & Werkman (3)].

Some new observations complicating fermentation are now at hand. It was first shown that growth and utilization of glucose by two strains of *Bact. acidi propionici* were very markedly accelerated by press juice from the potato or clover. This was still active when freed from protein and carbohydrate, while about one-third of the activity was shown by the ash. Fermentation of starch was less affected than that of glucose, while utilization of lactate was unaffected. The authors suggest that the activity is due to a hexokinase which governs the change from stable to active glucose (Fromageot & Tatum).

A comparable phenomenon has been disclosed regarding the balance of products in the butyric fermentation (Tatum, Peterson & Fred). Corn (maize) alone produces a low yield of butyl alcohol, which is raised thirty to one hundred fold by the addition of extracts of certain vegetable material (potato, yam, orange, lettuce, or cabbage), the yields of ethyl alcohol and acetone being unaltered. In the case of potato extract the active material was freed from proteins (by heat coagulation) and carbohydrate (ammonia with lead acetate) without loss.

A study of the anaërobic fermentation of glucose by thick suspensions of root-nodule bacteria (*Rhizobium trifolii*) in the presence of calcium carbonate gave mixed products characteristic of butyric fermentations. In the early stages considerable amounts of *dl*-lactic acid were found, but this disappeared later, giving place to more butyric acid. The molar ratio  $\text{C}_4\text{H}_8\text{O}_2 : \text{H}_2 : \text{CO}_2 = 1 : 2 : 1$  was found. Small quantities of ethyl alcohol and acetic acid were found, also some succinic acid which may have come from bacterial decom-

position; no formic acid or glycerol were present (Virtanen, Nordlund & Hollo).

Like yeast, several species of bacteria are now found to decompose phosphoglyceric acid into phosphoric and pyruvic acids: *Bact. delbrückii* in the presence of toluene (Neuberg & Kobel); *Bact. coli*, fresh and dried preparations [Antoniani (1)]; Timothy-grass bacillus (Cattaneo).

Growth experiments on *dl*-glyceric acid with *Bact. coli* and *Bact. lactis aërogenes* gave no lactic acid, but ethyl alcohol, formic acid, and acetic acid were produced; acetoin was formed by the latter organism only [Antoniani (2)].

The usual mixed fermentation obtained by *Bact. coli* can be displaced so as to become a predominantly lactic fermentation. This is done by using as substrate dilute solutions (0.38 per cent) of sodium hexosediphosphate (the hexose complex from which methyl glyoxal has been obtained), toluol and glutathione—the co-ferment of glyoxalase. By thus choosing conditions favouring the production and fermentation of methyl glyoxal, yields of lactic acid of 72 and 98 per cent from *Bact. coli* in wet and dry preparations, respectively, were obtained (Cattaneo & Neuberg).

The observations of Kempner on the inhibition of butyric acid fermentations (*Cl. butyricum*) by cyanide and carbon monoxide, reported last year,<sup>1</sup> have been extended. It is now shown that the inhibition by carbon monoxide is reversible by light, but that the intensity of light required is about 300 times greater than in the case of the *Atmungsferment*. It is shown that the dissociation constant,  $K$ , of the enzyme-CO compound increases with intensity of light,  $\Delta K$  ( $K$  in light —  $K$  in dark) being nearly proportional to light intensity. Light absorption by the enzyme-CO compound occurs from 650  $m\mu$  in the red to 360  $m\mu$  in the ultra violet (Kempner & Kubowitz). A third study on the same subject (Kubowitz) shows that the inhibitions by cyanide and carbon monoxide do not influence the primary sugar breakdown, but only the subsequent reactions by which butyric acid, carbon dioxide, and carbon monoxide are produced, the whole of the sugar now going over to lactic acid. Thus, in an atmosphere of argon alone, a pure butyric fermentation occurs ( $C_6H_{12}O_6 = C_4H_8O_2 + 2CO_2 + 2H_2$ ); in carbon monoxide alone a pure lactic fermentation occurs, while in argon + 5 per cent and

<sup>1</sup> *Ann. Rev. Biochem.*, 3, 525 (1934).

25 per cent carbon monoxide, respectively, mixed lactic and butyric fermentations are found.

A third inhibitor to the butyric fermentation is found to be hydrogen, but this is less complete than the other two, 2, 30, and 100 per cent hydrogen causing inhibitions of 18, 36, and 48 per cent, respectively. The fermentation of pyruvic acid by the same organism ( $\text{CH}_3 \cdot \text{CO} \cdot \text{COOH} = \text{CH}_3\text{COOH} + \text{CO}_2 + \text{H}_2$ ) is subject to similar inhibitions by carbon monoxide and hydrogen.

The ketogenic organism, *Bact. xylinum*, oxidises lactic acid to pyruvic acid; the equilibrium potential, lactic  $\rightleftharpoons$  pyruvic, is the same as that found by other workers for the same reaction (Cozic).

#### ENZYMES

The fibrinolysin of haemolytic streptococci has now been obtained in full activity in sterile cell-free filtrates from broth cultures of the organism. Fibrinolysis occurs both when the lysin is added to the preformed clot or if it is mixed with plasma or fibrinogen before clot formation. The latter is the better method, and was the one used in the experimental investigation. All the haemolytic streptococcal strains isolated from human patients work on human fibrinogen, but only three-eighteenths of those of animal origin. An interesting specificity is shown in the reaction; rabbit plasma clotted by rabbit thrombin is resistant to lysis by haemolytic streptococci of human origin, but rabbit plasma clotted by human thrombin is susceptible (Tillett & Garner).

The lysin was concentrated and partially purified by several methods: precipitation with three volumes of ice-cold alcohol, re-solution of the active precipitate in phosphate buffer at pH 7.2, concentration by vacuum dialysis, adsorption upon one of the polyaluminium hydroxides of Willstätter, and elution at pH 7.3. The concentrate is fairly heat-stable, but is slowly destroyed at neutrality between 75° and 100° and more quickly on either the acid or alkaline side (Garner & Tillett).

The nature of the lytic action seems to involve the early stages of proteolysis. Before lysis human fibrinogen is precipitated by 50 per cent saturation with sodium chloride and 25 per cent saturation with ammonium sulphate and is denatured at 57°. After fibrinolysis for sixty minutes, 50 per cent saturation with sodium chloride and 25 per cent saturation with ammonium sulphate cause no precipitation, but 35 per cent and 40 to 45 per cent saturation with ammonium

sulphate cause precipitation and flocculation, respectively. Fibrinolysin has no measurable hydrolytic effect on casein, gelatin, or peptone as measured by liberation of amino-nitrogen, but a slight effect is observed on solid human fibrin.

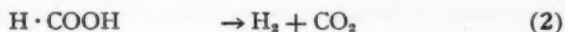
The plasma clot of patients convalescing from acute haemolytic streptococcal infections shows resistance to lysis; this resistance gradually disappears (Tillett, Garner & Edwards).

Pneumococcal haemolysin has been shown to be in the filtrate from the culture fluid. The amount is maximal when the total and viable counts are maximal, and remains so for about twenty hours, and then falls off owing to oxidation. The disappearance is prevented by anaërobic conditions and is reversed by hydrosulphite (Cowan).

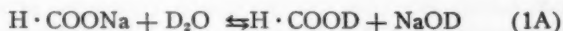
The plasma clot of patients with recurrent lymphangitis was found resistant to fibrinolysis by haemolytic streptococci (Morales-Otero & Pomales-Lebron).

Four organisms have been isolated from soil which decompose the specific carbohydrates of the pneumococcus, types I, II, and III, and also the non-specific carbohydrate (Sickles & Shaw). A single organism (a *Mycrococcus*) has been obtained from the soil which decomposes a number of specific polysaccharides, viz., those of *B. dysenteriae* Shiga and Flexner-γ, the pneumococcus type II and the tubercle bacillus (Morgan & Thaysen).

The mechanism of the action of formic hydrogenlyase has been attacked by a new weapon, viz., heavy water (Farkas, Farkas & Yudkin). The course of decomposition by this enzyme has hitherto been held to be



If these are the only reactions involved, then, if heavy water replaces  $\text{H}_2\text{O}$ , there would result<sup>2</sup>



<sup>2</sup> The symbol, D(=  $\text{H}^2$ ), is here used to denote the hydrogen isotope of mass 2 (deuterium).

That is, only the acid hydrogen atoms can be replaced by deuterium, and the deuterium content of the evolved hydrogen gas can never be more than half that of the original heavy water, and no deuterium molecules can occur. The enzymatic decomposition of sodium formate (by *Bact. coli*) in presence of heavy water of known deuterium content showed that the evolved gas always contained deuterium molecules ( $D_2$ ) and that the H/D ratio of the gas bore a constant relation, viz., 3.5, to the H/D ratio of the water. This is the equilibrium constant for the reaction,  $H_2O_{liq.} + HD \rightleftharpoons HOD_{liq.} + H_2$ , where  $K = \frac{[HOD][H_2]}{[H_2O][HD]} = 3.5$ . The analysis of the hydrogen evolved showed that  $H_2$ , HD, and  $D_2$  were always present in their equilibrium concentration:

$$K_1 = \frac{[HD]^2}{[H_2][D_2]} = 3.28 \text{ at } 40^\circ.$$

Hence the reactions 1A and 2A cannot occur, since deuterium molecules were present in the evolved gas, and the deuterium content of the gas in one case exceeded one-half the deuterium content of the water. Exactly similar results were obtained when a palladium catalyst replaced the *Bact. coli*. The authors suggest that atoms or radicals interact at the surface of the catalyst to account for the results obtained.

The bacterial origin of hydrogen sulphide has been considerably elucidated by Tarr. Following up the work previously reported<sup>3</sup> he has shown, by using washed suspensions of various sulphide-producing organisms on a series of pure sulphur compounds, that only  $\alpha$ -amino- $\beta$ -thiolcarboxylic acids and their -S-S- forms give hydrogen sulphide; 76 to 80 per cent of the theoretical yield was obtained in twenty-four hours. Substitution of the amino or carboxylic groups rendered the compounds almost, but not quite, immune from attack (2 per cent of hydrogen sulphide in twenty-four hours).

Other types of sulphur compounds gave no sulphide, e.g., methionine, mercaptides, thiourea. The enzyme concerned was found, *inter alia*, in *B. subtilis*, *Bact. coli*, *Cl. sporogenes*. Cultivation of the organisms on media to which 0.1 per cent cystine was added increased the amount of enzyme.

Nuclease has been found in *B. subtilis* and other members of the

<sup>3</sup> *Ann. Rev. Biochem.*, 3, 531 (1934).

group which decompose yeast nucleic acid to the extent of 48 per cent in forty-eight hours. The decomposition is confined mainly to the phosphoric acid ester of ribose and bases; some small amount of inorganic phosphate occurs, but this is probably attributable to acid hydrolysis (MacFadyen).

A detailed study of the methylene blue technique as applied to the dehydrogenases of *Bact. coli* shows that the reaction of this dye with different enzymes is not uniform. Thus, the poisoning effect of the dye on the enzyme is greatest for glucose dehydrogenase (80 per cent), next for succinic dehydrogenase (40 per cent), while formic dehydrogenase is unaffected. A proportionality between enzyme concentration and activity, in the case of glucose dehydrogenase, does not exist, the activity falling more rapidly than concentration. This observation led to the discovery that bacterial glucose dehydrogenase requires a coenzyme which is present in heated bacteria. It is replaceable by yeast cozymase, by the coenzyme of liver glucose dehydrogenase, and by Warburg's coenzyme of the system prepared from horse blood, which oxidises hexosemonophosphate. Moreover, bacterial coenzyme can replace liver coenzyme and cozymase. This bacterial coenzyme is present in other species of bacteria, both in those having glucose dehydrogenase and in *Bact. alkaligenes*, which lacks this enzyme (Yudkin [1, 2, and 3]).

#### PROTEOLYSIS

Bessey & King have studied the proteolytic activity of washed suspensions and cell-free filtrates of *Cl. sporogenes* and *Cl. histolyticum*. Both produce a proteolytic exo-enzyme capable of splitting proteins to polypeptides, that of *Cl. histolyticum* being more active. Washed cells have, in addition, deaminases, those of *Cl. sporogenes* being the more active; a marked specificity was shown, arginine and alanine being rapidly attacked, tyrosine and cystine only very slowly.

#### AMINO ACID BREAKDOWN

$\delta$ -Aminovalerianic acid has been isolated from *l*-oxyprolin by the action of mixed putrefactive bacteria in the presence of peptone and glucose; other possible intermediates, viz., hydroxy- $\delta$ -aminovalerianic acid and prolin, were not found (Keil & Günther).

Histamine has again been produced from *l*-histidine by freshly



isolated *Bact. coli*. The reaction took place in the presence of ammonium carbonate, 2.5 per cent glycerol, and 0.1 per cent lactose with a yield of 10 per cent. The same organism gave tyramine from tyrosine (Hirai).

#### TOXIN FORMATION

The isolation of an extremely interesting bacterial poison is reported from Batavia, Java. Frequent severe outbreaks of food poisoning among the native populations of this region have long been known. Prolonged laboratory and field work has now disclosed that the poison is produced aerobically on certain coconut products ("bongkrek" and "semaji") which have become infected with a hitherto undescribed bacillus. The poison was finally obtained on laboratory media containing glycerol and isolated as a yellow crystalline product (m.p. about 200°). It is nitrogenous, soluble in water, alcohol, and fat, less easily in benzene, chloroform, and ethyl acetate, and insoluble in petroleum ether. It forms an addition product with bisulphite and is reversibly oxidised and reduced. It is toxic in doses of 5γ when given intraperitoneally to rats and (probably) in doses of 0.5 mg. *per os* to man. The chemical composition of this body is not yet known, but a relationship with Warburg's yellow pigment and with lactoflavine is suggested (van Veen & Mertens; Mertens & van Veen).

#### GROWTH

A study of growth stimulants produced by yeasts (Pulkki) has cleared a lot of mist from that hazy field. A clear analytical and critical summary of previous work, followed by a series of carefully planned and controlled experiments with one strain of *B. mycoides*, show that for this organism a number of substances hitherto suspected of stimulating growth fail to do so; among these are ascorbic acid, yeast ash, and extracts of the same bacterium. Yeast extracts, on the contrary, increased growth 100 to 400 per cent. It was found that, when the number of cells was largely increased, the size was diminished, i.e., the stimulus is on cell division. The factors influencing the production of the stimulating materials in different strains of yeast have been studied. The contribution is particularly valuable as a model of technique and controlled experimentation.

Work on the oxidising (anaerobic) and reducing systems of the

cell has been extended by Aaron working with *Bact. paratyphosus B.* He has made a systematic study of substances which can act as hydrogen donators, and shown which of these can, in addition, function as sole sources of carbon in both aërobic and anaërobic growth. As in cases previously studied, certain compounds are oxidised by the cell which cannot function as sole sources of carbon for growth; such are formate, ethyl alcohol, glycine. As hydrogen acceptors the following are activated: nitrate, fumarate, malate, aspartate, and asparagin, the same as previously found for other facultative anaërobes. On a solid medium the anaërobic growth requirements are higher than on a liquid medium; thus, in the latter, with nitrate as acceptor and lactate as donator, ammonium chloride can supply the nitrogen requirements, while, in the former, one of the following also is necessary: leucine, serine, asparagin, aspartate, glutaminate, lysine, arginine, phenylalanine, tryptophane, histidine, proline. Glycine and alanine are inactive. In addition, serine seems to play the part of a hydrogen acceptor.

Metabolic studies on *Esch. coli* have been continued (Walker, Winslow & Mooney). In a peptone medium saturated with air, the cell multiplication is enormously higher than in nitrogen (655 and 32 millions per cc., respectively). Glucose raises the growth slightly on an aërated medium (861 millions per cc.) but much more on the anaërobic medium (i.e., from 32 to 142 millions per cc.). The output of carbon dioxide per cell per hour is almost unaffected by sugar aërobically, and anaërobically the effect is more marked; in peptone, carbon dioxide production attains a maximum of  $68 \times 10^{-11}$  mg. per cell per hour in the third hour, and then falls off, while in glucose it mounts to 211 and  $188 \times 10^{-11}$  mg. in the fourth and fifth hours, greatly surpassing the output under aërobic conditions. This appears to be another example of aërobiosis inhibiting fermentation.

From work with seven strains of *Cl. botulinum* it appears that acid digests of casein or gelatin will not support growth, but that a supplement of either tryptophane or cystine renders the medium adequate; sixteen other amino acids were tested, and none had the same effect as the two above-mentioned (Burrows).

The intracellular formation of fat has been studied with *B. prodigiosus*. Lipoid synthesis is favoured by liquid as opposed to solid media. The following substances when added to a liquid meat medium raise the lipid content of the organism sometimes as much as 400 per cent: ethyl alcohol, glycerol, glucose, sucrose, and mannitol. The

optimum temperature for lipid formation is 10° [Gorbach & Sablatnög (1 and 2)].

#### COLOURED SULPHUR BACTERIA

A paper by Gaffron (1) on this subject should have been reported in volume III of this Review, as it was published shortly after that of Muller and bears on the same subject. This worker actually proves manometrically what Muller showed indirectly by his carbon balance sheet, viz., that the bacterial cell (in this case *Rhodobacillus*), when irradiated anaerobically in the presence of fatty acids, absorbs carbon dioxide and at the same time grows, again proving that under the influence of light carbon dioxide acts as a hydrogen acceptor. The carbon dioxide absorbed depends on the amount of the fatty acid, and also on the length of the chain. Thus the ratio, mols CO<sub>2</sub> absorbed/mols fatty acid used, was found equal to 0.89, 1.42, 1.40, 1.87, 2.34, 2.40, 2.96, 3.50 on rising from acetic acid to nonylic acid, giving an average of 0.50 for each additional carbon atom. The reduction value of the carbon compound also affects this ratio, the values for butyric and crotonic acids being 1.40 and 1.06, respectively.

Nitrate partially inhibits carbon dioxide reduction, being itself reduced to nitrite.

Gaffron adds another important item to our knowledge of these organisms. He finds that *Thiocystis* (a characteristic red sulphur bacterium) cannot replace hydrogen sulphide (and other oxidisable sulphur compounds) by organic material, differing in this respect from the purple bacteria, e.g., *Rhodovibrio*. Thus, if a suspension of the latter is irradiated in the presence of carbon dioxide and butyrate, the carbon dioxide is assimilated and the carboxyl group reduced; with *Thiocystis* nothing occurs, although the latter grows well anaerobically on a medium with carbon compounds in place of hydrogen sulphide. The explanation lies in a reaction occurring anaerobically in the dark and independent of carbon-dioxide assimilation, whereby *Thiocystis* reduces sulphate by means of butyrate to hydrogen sulphide, giving carbon dioxide and organic acids. When the organism is again irradiated the sulphide reduces the carbon dioxide in the characteristic manner.

Spectrum analysis shows that, in the system *Thiocystis*, thio-sulphate, and carbon dioxide, irradiation with ultraviolet light (120 to 900 mμ) causes rapid absorption of carbon dioxide; red light (620 to

700 m $\mu$ ) is less active, and blue light (420 to 450 m $\mu$ ) is inactive [Gaffron (2)].

### NITROGEN FIXATION

Burk and his school have further added to our knowledge of the nitrogen-fixing enzyme system of *Azotobacter*. To clarify conceptions the following nomenclature is now adopted:

"Azotase" (A) is the total enzyme system catalysing the fixation of nitrogen. The enzyme in the system combining with nitrogen is "nitrogenase" ( $A_{N_2}$ ); the calcium and strontium components are  $A_{Ca}$  and  $A_{Sr}$ ; the component requiring a minimum hydroxyl concentration is  $A_{OH^-}$ , and the inactive form of the component below pH 6 is  $A_H^+$ .

It is shown (Burk, Lineweaver & Horner) that the fixation of free nitrogen attains a maximum at pH 7.8 and falls to zero at pH 6.0 in a curve which is concave downwards; the inactivation is reversible above pH 5.0, but irreversible below this. The rate of consumption of fixed nitrogen follows a different curve, and approaches zero asymptotically and is reversible above pH 4.5. The limiting pH value of 6.0 for fixation is independent of any factor known to influence the fixation process, i.e., calcium or strontium concentration and nitrogen pressure. The Michaelis constant for the fixation process (i.e., pressure of nitrogen at 0.5 maximum velocity) is independent of pH and calcium and strontium concentration.

Phase rule analysis (Burk & Lineweaver) of azotase activity as a function of pH discloses a two-component heterogeneous system with three phases in equilibrium at the critical pH: aqueous, active non-aqueous, and inactive non-aqueous; the active basic component exists above the critical pH (6.0), and the inactive acidic one below.

Mathematical treatment of previous data (Lineweaver, Burk & Deming) on the fixation of nitrogen as a function of nitrogen pressure indicates that one nitrogen molecule combines reversibly with one enzyme molecule ( $E$ ), nitrogenase, giving the complex  $N_2E$ , whose thermodynamic dissociation constant,  $K_{N_2}$ , equals  $[E] \cdot [N_2]/[N_2E]$  which equals  $21.5 \pm 0.2$  vol. per cent. This constant is independent of the following factors influencing the fixation process: concentration of calcium, strontium, and oxalate, pH, and concentration of iron.

The necessity for molybdenum in fixation has been confirmed. This element works at extremely low concentrations, the optimum concentration being 100 parts per trillion while an observable effect is apparent at 1 to 3 p.p.t. Molybdenum can be replaced by vanadium, whose minimum and optimum concentrations are 200 to 500 per cent higher. Neither of these elements affects growth on fixed nitrogen. Iron and magnesium, on the other hand, affect growth in both free and fixed nitrogen (Burk).

#### MARINE BIOLOGY

Two papers of great interest on the part played by marine bacteria in the life cycle in the sea are difficult to summarise and must be consulted in the original (Waksman, Carey & Reuszer; Waksman, Hotchkiss & Carey).

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